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# CYANIDE-SENSITIVE BACTERIAL RESPIRATORY SYSTEMS DIFFERENT FROM THE USUAL CYTOCHROME-CYTOCHROME OXIDASE SYSTEM\*

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(Received for publication May 22 1942)

In previous communications (Sevag and Shelburne, 1942) it was shown that sulfonamide drugs inhibited the aerobic respiration and anaerobic glycolysis of *Streptococcus pyogenes* and the respiration of pneumococcus Type 1. The inhibition of respiration could take place in the absence of growth. In the presence of growth the inhibition of respiration and of growth occurred to the same relative extent. It is therefore, evident that the sulfonamide drugs interfered primarily with the respiratory enzyme systems of the bacteria studied.

To determine which enzyme systems are inhibited by the sulfonamide drugs, it is necessary to learn the nature of the catalysts associated with the respiratory functions of these organisms.

The present study shows that *Streptococcus pyogenes* and pneumococcus Type 1 contain cyanide sensitive respiratory systems which are different from the usual cytochrome *c*-cytochrome oxidase system.<sup>1</sup> The respiratory systems of these bacteria appear to be similar to that found by Keilm and Harpley (1941) in *B. coli*.

## EXPERIMENTAL RESULTS

*Inhibition of the Aerobic Respiration of Streptococcus pyogenes and Pneumococcus Type 1 by KCN, NaN<sub>3</sub>, and Na<sub>2</sub>S* The measurement of respiration of streptococcus and pneumococcus was carried out as described in the previous communications (Sevag and Shelburne, 1942). Glucose was used as substrate. The results of numerous experiments in the presence of KCN are given in Table I.

The results show that the respiration of all of the virulent and non virulent strains of *Streptococcus pyogenes* and pneumococcus Type 1 is strongly though not completely inhibited by 0.01 to 0.0001 M KCN.

\* This work was started under a grant from The Commonwealth Fund and continued under grants from The Josiah Macy Jr. Foundation and Merck and Co.

<sup>1</sup> In addition to the presence of cyanide-sensitive respiratory systems, *Streptococcus pyogenes* and pneumococcus Type 1 appear to contain also a cyanide-insensitive system (possibly dehydrogenase-cozymase-flavoprotein system). This is borne out by the fact that aerobic respiration of these bacteria is not completely inhibited by poisons known to be specific inhibitors of heme type of enzymes.

In experiments with pneumococci the presence of 0.01 M pyruvate increased the respiration by 25 per cent. This increase in the respiration is not due to the metabolism of pyruvate as substrate but is due to the removal by pyruvate

TABLE I

*Inhibition of the Aerobic Respiration of Streptococcus pyogenes and Pneumococcus Type 1 by Potassium Cyanide*

Experiments	Bacterial strain	Bacterial cells per test	C mm O <sub>2</sub> consumed during 2 hrs Normal respiration	Per cent inhibition by KCN		
				0.01 M	0.001 M	0.0001 M
		mg				
1	C203M	1.39	158	65	53	—
2	"	1.13	234	—	74	43
3	"	0.72	182	—	65	52
4	"	1.50	174	—	67	47
5	"	1.27	165	83	83	40*
6	"	1.73	68	88	82	60
7	"	3.48	257	90	88	73
8	C203S	0.91	91	—	46	24
9	"	0.91	97	—	42	31
10	"	0.83	156	—	32	38
11	C203R	0.94	61	—	26	18
12	"	—	120	—	50	17
13	"	2.76	186	69	65	51
14	1685M	1.54	215	—	63	33
15	"	1.12	151	—	63	—
16	1685S	1.54	153	—	60	39
17	"	0.76	124	—	66	45
18	1048M	1.91	341	—	60	26
19	"	1.91	346	—	59	34
20	Pneu Type 1	0.90	514	—	40	19
21	"	2.50	844	71	64	13
22	"	1.83	904	70	63	14

\* 0.00001 M concentration of KCN gave 33 per cent inhibition

Each vessel contained 0.4 cc of 0.1 M glucose, KCN as designated, 0.5 cc yeast extract, 0.5 cc normal horse serum, 0.4 cc of twice washed organisms (4 hr cultures), and the total volume made to 5.8 cc. with phosphate buffer of pH 7.3. 0.3 cc of 20 per cent KOH was placed in the alkali tube. Controls containing no organisms were run on all vessels and the necessary corrections made.

Experiments 6, 7, 13, 22, and 23 contained in the alkali tube of the Warburg vessel a KOH-KCN mixture which gave the same partial pressure of HCN as that of the mixture studied (Krebs, 1935).

M = mucoid, S = smooth, R = rough strains of *Streptococcus pyogenes*

of the toxic H<sub>2</sub>O<sub>2</sub> formed during the oxidation of glucose (Sevag, 1933). The inhibition of the respiration by cyanide in the presence of pyruvate was reduced from 52 to 38, and from 32 to 20 per cent, with 0.004 M and 0.001 M KCN respectively.

The results of the experiments on the inhibition of the aerobic respiration of *Streptococcus pyogenes* and pneumococcus Type 1 by sodium azide are given in Table II. The experiments were carried out at pH 5.9 to liberate the maximum amount of hydrozoic acid. It will be seen that 0.01 to 0.0001 M sodium azide strongly inhibited the respiration of both organisms. Pneumococcal respiration was inhibited to a greater extent than that of streptococci. The results of experiments (not given in Table II) carried out at pH 7.4 likewise showed that 0.01, 0.001, and 0.0001 M sodium azide inhibited streptococcal respiration, 76, 53, and 15 per cent, respectively, and that of pneumococcus 89, 31, and 13 per cent, respectively. Inhibition with sodium azide appeared to be more pronounced at lower pH values.

TABLE II

*Inhibition of the Aerobic Respiration of Streptococcus pyogenes and Pneumococcus Type 1 by Sodium Azide*

Strain of organism	Bacteria per test	Cmm O <sub>2</sub> consumed during 1 hr. Normal respiration	Per cent inhibition by NaN <sub>3</sub>		
			0.01 M	0.001 M	0.0001 M
C203M	mg				
	1.73	21	100	60	0
	3.48	88	92	80	33
C203R	2.74	80	—	35	33
Pneum.	2.18	719	97	97	58
"	2.12	571	94	97	74

Each vessel contained 0.4 cc. of 0.1 M glucose, NaN<sub>3</sub> as designated, 0.5 cc. yeast extract, 0.5 cc. normal horse serum, 0.4 cc. of 4 hr. twice washed organisms, and the total volume made to 5.8 cc. with phosphate buffer at pH 5.9. 0.3 cc. of 20 per cent KOH was placed in the alkali tube of the vessel. Controls containing no organisms were run on all vessels and the necessary corrections made.

At pH 7.73, 0.01 M and 0.001 M sodium sulfide inhibited the respiration of *Streptococcus pyogenes* (C203M) 50 and 60 per cent, respectively. Under identical conditions the inhibition of the respiration of pneumococci with sodium sulfide was, respectively 33 and 8 per cent. The results of experiments carried out at pH 7.4 were similar.

*Reversibility of the Aerobic KCN Inhibition*—A suspension of *Streptococcus pyogenes* was treated with 0.001 M KCN for 30 minutes, centrifuged, and the supernatant discarded. The bacteria were then washed in phosphate buffer and suspended in phosphate buffer solution. The activity of the suspension was determined in the presence and in the absence of KCN. A control suspension which had not received any treatment with KCN was used for comparison. The respiration of the suspension which had been treated with KCN and washed showed no appreciable difference from that of the untreated control.



*Inhibition of the Anaerobic Glycolysis of Glucose in Pneumococcus Type 1* — The anaerobic glycolysis of glucose in *Streptococcus pyogenes* was not inhibited by either KCN or  $\text{NaN}_3$  in three out of four experiments. In contrast, the anaerobic glycolysis of glucose in pneumococcus Type 1 was inhibited by KCN (Table III). (The inhibition with 0.01 M sodium azide was 21 per cent.)

*Experiments on the Presence of Cytochrome c-Cytochrome Oxidase System* — The strong inhibition of the aerobic respiration of *Streptococcus pyogenes* and pneumococcus Type 1 by well known heme poisons such as KCN,  $\text{NaN}_3$ , and  $\text{Na}_2\text{S}$  would suggest that the respiration is in part catalyzed by the heme type of oxidase system. We were, however, unable to demonstrate the presence of cytochrome *c* in these organisms using a pocket spectroscope with which the

TABLE III

*Inhibition of the Anaerobic Glycolysis of Glucose by Pneumococcus Type 1 with Potassium Cyanide*

Organism	Bacteria per test	C.mm $\text{CO}_2$ evolved during 2 hrs Normal glycolysis	Per cent inhibition by KCN			
			0.001 M	0.0005 M	0.0002 M	0.0001 M
1 Pneumococcus I	mg 3.00	1632	40	28	16	0
2 "	3.00	1615	40	22	14	15
3 "	0.99	1920	35	25	12	6
4 "	0.99	1565	26	17	16	14

Each vessel contained 0.27 cc of 0.2 M glucose, 0.27 cc of 1 M  $\text{NaHCO}_3$ , KCN as designated, 0.5 cc of yeast extract, 0.5 cc normal horse serum, 0.4 cc of twice washed organisms (4 hr culture), and the total volume made to 5.8 cc with 0.9 per cent NaCl solution. The system was adjusted to a pH of 7.3 with a glass electrode. Systems 2 and 4 contained also 0.2 cc of M/15 phosphate buffer of pH 7.3. The experiments were carried out at 37.5°C in an atmosphere of 95 per cent  $\text{N}_2$  and 5 per cent  $\text{CO}_2$ .

*c* bands of bakers' and brewers' yeast could be seen easily. We were also unable to demonstrate the presence of cytochrome *c* by measuring, manometrically and colorimetrically, the oxidation of *p*-phenylenediamine by streptococcus and pneumococcus. Our results suggest that these bacteria do not catalyze the  $\text{O}_2$ -uptake by *p*-phenylenediamine. The addition of cytochrome *c* to the systems did not influence the reaction.

It is interesting to note that *B. subtilis* and *B. proteus*, known (Stephenson, 1939) to contain cytochrome *c*, also usually do not catalyze the oxygen uptake of *p*-phenylenediamine.<sup>2</sup> In contrast, *B. pyocyaneus* (*Pseudomonas aeruginosa*) and yeast oxidized *p*-phenylenediamine readily.

<sup>2</sup> According to Frei, Reidmüller, and Almásy (1934) *B. pyocyaneus* contains *a*, *b*, and *c* cytochrome components and gives a colorimetric cytochrome oxidase test. In contrast, their results with *B. subtilis* were irregular. Though certain cultures of *B. subtilis* spectroscopically showed cytochrome components *a*, *b*, and *c*, and gave a

Keilin (1929) showed that heated yeast contains an oxidase system which oxidizes *p*-phenylenediamine giving a dark purple merquinoid salt. Keilin and Harpley (1941) stated that a positive reaction is obtained only in the presence of both *a*<sub>2</sub> and *c* components. Using Keilin's method (with slight modification) we were able to demonstrate that *Streptococcus pyogenes*, *Escherichia coli*, pneumococcus Type 1, and *Staphylococcus aureus*, which do not contain cytochrome *c*, fail to give positive color reactions with *p*-phenylene diamine. On the other hand, yeast, *B. subtilis*, and *B. pyocyaneus* which contain all the components of cytochrome reacted positively. The only exception to this latter group was *B. proteus* which gave negative results (see discussion).

*Streptococcus pyogenes* and pneumococcus likewise did not catalyze the O<sub>2</sub>-uptake by hydroquinone (0.02 M) as a substrate for the cytochrome-cytochrome-oxidase system.

Streptococci but not pneumococci catalyzed the oxygen uptake in the presence of a 0.02 M solution of cysteine. A suspension of streptococci in a control vessel consumed only 5 c. mm. of O<sub>2</sub> during 90 minutes at 37.5°C. In the presence of cysteine at pH 6.9, 37 c. mm. of O<sub>2</sub>, and in another experiment 67 c. mm. of O<sub>2</sub> were consumed, an increase of 5- to 12 fold in O<sub>2</sub>-uptake (these values are corrected for the auto-oxidation of cysteine).

**Tests for Copper Protein Enzymes and Xanthine Oxidase**—*Streptococcus pyogenes* and pneumococcus Type 1 failed to catalyze oxygen uptake in the presence of tyrosine, adrenalin, pyrocatechin, xanthine, and hypoxanthine showing that these organisms do not contain either copper protein enzymes or xanthine oxidase.

**A Thermostable System with Peroxidase Activity**—It is known that numerous aerobic bacteria contain a thermostable peroxidase (Callow, 1926; Keilin, 1929). Farrell (1935) showed that hemolytic streptococci contained a thermostable peroxidase, though spectroscopically he could not find peroxidase bands in streptococcal suspensions. In view of the fact that  $7.2 \times 10^{-4}$  mg./cc. of hemin ( $1.1 \times 10^{-7}$  M) gives a positive peroxidase test (unpublished observation), the negative spectroscopic results do not indicate that the thermostable perox

weak colorimetric cytochrome oxidase test the cultures of three strains (i.e. Marburg Hutten and Michigan) of *B. subtilis* in numerous spectroscopic tests showed only *a* and *b* cytochrome components and failed to give cytochrome oxidase tests. They stated that the presence and absence of cytochrome oxidase and the cytochrome components are controlled by (a) the age and the degree of spore formation (b) specific characteristics of the strains and (c) certain unknown factors controlling the formation of cytochrome during growth. *B. proteus* contained cytochrome components *a* and *b*, and failed to give the cytochrome oxidase test. A strain of *Streptococcus hemolyticus* contained neither cytochrome components nor gave a cytochrome oxidase test.

idase in bacteria is not of the hematin type. Using Farrell's technique we obtained a positive peroxidase reaction with boiled and unboiled, aerated and unaerated suspensions of *Streptococcus pyogenes*, pneumococcus Type 1, *E. coli*, and normal horse serum. Even though the serum contained no visible hemolyzed blood, the positive peroxidase reaction which it gave would indicate the presence of traces of hematin derivative.

#### DISCUSSION

Fujita and Kodama (1934) reported that cyanide and CO did not inhibit the respiration of *Streptococcus hemolyticus*, *Streptococcus viridans*, and pneumococcus Types 1, 2, and 3. Neither the streptococci nor the pneumococci were stated to contain any of the cytochrome components. Tamiya and Yamaguchi (1933) on the other hand, reported the presence of cytochrome bands in *Streptococcus erysipelatis* and in pneumococcus Type 1. Barron and Jacobs (1938) showed that 0.01 M HCN inhibited the oxidation of glucose by three strains of hemolytic streptococci, while three other strains were found to be insensitive to cyanide. These results were emphasized to show that different strains of hemolytic streptococci exhibited different metabolism. They also showed that a strain of streptococcus which was insensitive to HCN became partially sensitive to it on repeated transplantation over a period of 8 months.

In our experiments the aerobic respiration of *Streptococcus pyogenes* and pneumococcus Type 1 was strongly inhibited by KCN,  $\text{NaN}_3$ , and  $\text{Na}_2\text{S}$ . The results with pneumococcus were in contrast to those found previously (Sevag, 1933) with another strain of pneumococcus Type 1 which was insensitive to cyanide. It was also shown (Sevag, 1933, Sevag and Maiweg, 1934) that two strains of pneumococcus belonging to the same type exhibited different metabolism. These findings show that our observations in this respect are in agreement with those of Barron and Jacobs (1938).

This inhibition of the respiration of *Streptococcus pyogenes* and pneumococcus Type 1 by heme poisons might indicate a cytochrome *c*-cytochrome oxidase catalysis of the respiratory processes of these bacteria. However, these bacteria were incapable of oxidizing *p*-phenylenediamine which is known to be a specific substrate for the cytochrome *c*-cytochrome oxidase system. Except for an occasional weak catalysis of the oxygen uptake of *p*-phenylenediamine the results with *E. coli*, *Staphylococcus aureus*, and *B. proteus* were likewise negative. Oxygen uptake measurements with *B. subtilis* in the presence of *p*-phenylenediamine gave also negative results. These results are interesting in view of the fact that both *B. proteus* and *B. subtilis* are reported to contain cytochrome *c*. Yeast cells invariably catalyzed this reaction. Addition of cytochrome *c* to the above bacterial and yeast reaction systems did not accelerate their reaction.

The oxidation of *p*-phenylenediamine to dark purple merquinoid salts on the other hand, was observed, with one exception, with all the organisms which have been reported to contain cytochrome *c*, and negative results were obtained with the organisms lacking it. However, *B. proteus* which contains cytochrome *b* and *c* components and lacks component *a* failed to give a positive test. Pneumococcus Type 1, reported to contain cytochrome components *a* and *b*, but lacking component *c* likewise did not oxidize *p*-phenylenediamine.

The above results show that *Streptococcus pyogenes* and pneumococcus Type 1 contain cyanide-sensitive systems which are different from the usual cytochrome *c*-cytochrome oxidase systems. Recent experiments by Keilin and Harpley (1941) provide enzymatic and spectroscopic evidence for this view. *B. coli* manifested identical respiratory behavior in the presence of cyanide. According to these authors, *B. coli* showed spectroscopically the presence of cytochrome components  $n_1$  and  $a_2$ , while *a*, *b*, *c*, the characteristic bands of the cytochrome system in yeast cells, were absent. According to Keilin and Harpley (1941) a complete cytochrome *c*-cytochrome oxidase system must be present to oxidize *p*-phenylenediamine. This involves the presence of components *c* and *a* and  $a_2$ . The last component is cytochrome oxidase proper. Component  $a_2$  is not always spectroscopically visible but apparently invariably present in systems containing component *a*. In the light of the above interpretation the inability of *B. proteus* to oxidize *p*-phenylenediamine can be explained. This organism, though it contains cytochrome *b*, and *c*, lacks the component *a*, and apparently the fourth component  $a_2$  which is considered essential for the oxidation of cytochrome *c*, and therefore of *p*-phenylenediamine. Similarly the inability of pneumococcus and *Staphylococcus aureus* to oxidize *p*-phenylenediamine is apparently due to the absence of cytochrome *c* even though the components *a* and *b*, and assumedly  $a_2$  are present. The respiratory system of *Streptococcus pyogenes* appears to be similar to pneumococcus and *Staphylococcus aureus*. This requires further investigation.

Wieland believed and sought to show that  $H_2O_2$  is one of the products of aerobic biological oxidation reactions. While he was able to demonstrate readily the formation of  $H_2O_2$  in cyanide-insensitive systems he failed to show this with systems containing heavy metal catalysts for the reason that catalase invariably was present. Selective inhibition of catalase without inhibiting the respiration was not possible (Wieland and Sevag, 1933). The results of the present study with the strain of pneumococcus Type 1 which forms hydrogen peroxide (Sevag and Shelburne, 1942) appear to support Wieland's point of view.

Both *B. coli* and pneumococcus Type 1 appear to contain identical cyanide sensitive respiratory enzyme systems. However, while the formation of hydrogen peroxide in pneumococci, which lacks catalase, is easily demonstrable, the formation of hydrogen peroxide in *B. coli*, which contains catalase, cannot

be demonstrated. As far as we know, the formation of hydrogen peroxide in a cyanide-sensitive heme type of enzyme system has not previously been demonstrated.

Since the previous experiments showed that sulfonamide drugs inhibited the respiration and growth of bacteria (Sevag and Shelburne, 1942, Sevag, Shelburne, and Mudd, 1942) to the same extent, a better knowledge of the nature of the respiratory enzymes appeared to be necessary to elucidate the difference in the susceptibility of various bacteria to the action of sulfonamide drugs. It was found that in all the bacteria studied above the respiration was catalyzed by cyanide-sensitive systems of heme type. However, while some of these bacteria contained the whole cytochrome-cytochrome oxidase system, the others contained different cytochrome systems.

#### SUMMARY

Aerobic respiration of *Streptococcus pyogenes* and pneumococcus Type 1 are strongly inhibited by KCN,  $\text{NaN}_3$ , and  $\text{Na}_2\text{S}$ . The anaerobic glycolysis of glucose by pneumococcus is also inhibited by KCN and  $\text{NaN}_3$ .

*Streptococcus pyogenes*, *E. coli*, pneumococcus Type 1, *B. subtilis*, *B. proteus*, and *Staphylococcus aureus* did not catalyze the oxygen uptake by *p*-phenylenediamine in the presence of added cytochrome *c* or in its absence. Yeast cells, *B. subtilis*, and *B. pyocyaneus* oxidized *p*-phenylenediamine to a dark purple merquinoid substance in contrast to the other bacteria mentioned above.

*Streptococcus pyogenes* in contrast to pneumococcus Type 1 catalyzed the oxygen uptake by cysteine. Neither of these bacteria catalyzed the oxygen uptake by tyrosine, adrenaline, pyrocatechin, xanthine, and hypoxanthine.

*Streptococcus pyogenes*, pneumococcus Type 1, and *E. coli*, boiled and not boiled, gave positive peroxidative tests with benzidine showing the presence of hematin compounds.

The results discussed in the light of the interpretations offered by Keilin and Harpley show that *Streptococcus pyogenes* and pneumococcus Type 1 contain cyanide-sensitive respiratory systems which are different from the cytochrome *c*-cytochrome oxidase system.

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# THE ANTIGENICITY OF *d* RIBONUCLEASE, THE INHIBITION OF THE ENZYME BY ITS HOMOLOGOUS IMMUNE SERUM\*

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(Received for publication, May 28, 1942)

A thermostable enzyme capable of digesting *d* ribose (yeast) nucleic acid was first described by W Jones in 1920 (1). A partial purification of this enzyme with acetone was carried out by Dubos and Thompson who called the enzyme ribonuclease (2). Kunitz described the preparation and properties of a crystalline protein he isolated from beef pancreas which appeared to be the same as the ribonuclease. He provisionally called this material ribonuclease. This crystalline preparation has a molecular weight of about 15,000 (3).

Enzymes have been reported to act as antigens. However, these enzymes all have had a molecular weight of at least 35,000. It appeared of interest to determine whether an enzyme of this small molecular weight (15,000) could be antigenic. Whether the activity of the enzyme could be inhibited by its combination with specific antiserum also offered an interesting problem.

## Materials Used

The *d* ribonuclease preparations used in this communication were kindly supplied by Dr. M. Kunitz to whom we are greatly indebted. Five different ribonuclease preparations have been used: a crude preparation, and products crystallized respectively three, five, six and eight times. Methods for the preparations of the crystallized ribonuclease as well as some of its properties are given by Kunitz (3).

The *d*-ribose (yeast) nucleic acid used was a Pfanstiehl commercial product which was purified in this laboratory by a method using  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{CHCl}_3$  as previously described (4) to give a phosphorus content of 9.3 per cent (King's modification (5) of Fiske and Subbarow (6)).

## EXPERIMENTAL

### Immunization Experiments

Antisera against the purified crystalline preparation of ribonuclease were prepared by three different methods.

(a) Intramuscular injection of ribonuclease adsorbed on aluminum gel after the method of Hektoen and Welker (7). A single injection of 110 mg. of crystalline ribonuclease adsorbed on aluminum gel was given. The amount of ribonuclease in the

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gel was determined by nitrogen analysis (Kjeldahl method) (b) Intravenous injections of the preparation used in (a) (c) Intravenous injections of saline solutions of crystalline ribonuclease

Antibodies were demonstrated following immunization by all of the above methods Tests for the precipitation were set up by mixing 0.2 ml of antigen in the proper dilution, with 0.2 ml of antiserum These tubes were placed in the 37°C

TABLE I  
*Specificity of Antiserum*

Antisera vs	Cattle serum							Crude RN						6 × purified RN				
	1 10	1 50	1 250	1 1,250	1 6,250	1 31,000	1 150,000	1 500	1 2,500	1 12,500	1 62,500	1 312,500	1 1.5 × 10 <sup>6</sup>	1 62,500*	1 125,000	1 250,000	1 10 <sup>6</sup>	1 2 × 10 <sup>6</sup>
RN (6 × purified)	0	0	0	0	0	0	0	0	0	0	1	2	0	1	2	3	1	0
RN (crude) No 1	0	0	0	0	0	0	0	0	1	2	1	0	0	0	1	3	1	0
RN (crude) No 2	0	0	0	0	0	0	0	0	0	1	2	0	0	0	4	3	1	0
Cattle serum No 1	4	3	3	3	1	±	0	0	0	0	0	0	0	0	0	0	0	—
Cattle serum No 2	4	3	3	3	2	±	0	0	0	0	0	0	0	0	0	0	0	0
N R S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

RN = Ribonuclease 1 = +, 2 = ++, 3 = +++, 4 = ++++

\* All dilutions up to 62,500 are negative

TABLE II

*Reactivity of Antiserum against Five Times and Eight Times Crystallized Enzyme Preparations*

Antisera	Antigen	Antigen dilution					
		1:100	1:1000	1:10 <sup>4</sup>	1.5 × 10 <sup>4</sup>	1:10 <sup>5</sup>	1.5 × 10 <sup>5</sup>
5 × RN*	5 × RN*	0	sl tr	4	4	2	0
5 × RN*	8 × RN†	0	0	4	4	2	tr
8 × RN No 1	8 × RN	—	—	2	4	3	1
8 × RN No 2	8 × RN	—	—	0	0	2	1

RN = Ribonuclease

\* = 5 times crystallized ribonuclease

† = 8 times crystallized ribonuclease

water bath for 1 hour and in the refrigerator overnight The tubes were centrifuged before reading

The results obtained with the two methods of intravenous injections were the same Antibodies were demonstrable after a course of about thirteen injections (three times weekly) totalling about 30 mg of the purified crystalline ribonuclease As seen from Table I the reaction was positive up to an antigen dilution of one million Ordinarily there persisted a prozone which with some

antisera went up as high as to 1:100,000 antigen dilution. However, several antisera were prepared which did not show any antigen prozone. Also some of the antisera reacted more strongly than others. Antisera prepared against the five times crystallized enzyme preparation when tested serologically gave identical results against both the five times and eight times crystallized enzyme preparations. (See Table II.)

The crude ribonuclease was injected intravenously into rabbits, using 0.5 mg of material at the start. Antibodies against the crude preparation could be demonstrated after a total of 9 mg had been injected (7 injections). No reaction with this antiserum could be demonstrated against the crystalline ribonuclease. However, longer periods of immunization with the crude material gave precipitation against the crystalline preparation in dilutions up to one million. There was a definite prozone which in some cases extended to over 100,000 dilution of antigen. The crude ribonuclease, in dilutions of 2,500 to 62,500, gave precipitation against crude ribonuclease antisera. (See Table I.)

For controls, since the source of the enzymes was beef pancreas, rabbits were injected with cattle serum. Cattle antisera gave positive reactions with no prozone up to homologous antigen dilution of 31,000. No cross-reaction was given with any of the preparations of ribonuclease. The cattle serum, likewise gave no reaction against antiribonuclease sera (Table I). Normal rabbit sera when tested against the various ribonuclease preparations gave no reaction in any case.

#### *Action of Specific Antisera on the Enzyme Activity*

The amount of the purified enzyme preparation in the antigen-antibody precipitate was determined in the following manner. To varying amounts of the crystalline enzyme (in a volume of 0.2 ml.) was added 3 ml. of homologous antiserum. These tubes were shaken, allowed to stand in a 37°C. water bath for 1 hour, and in the refrigerator overnight. In the morning the tubes were centrifuged, the sediment washed with cold 0.85 per cent NaCl solution, and the precipitates analyzed for nitrogen. A curve was plotted using milligrams of enzyme as the abscissa and milligrams of protein precipitated as the ordinate. (See Fig. 1.) Obviously, the most satisfactory region to carry on this type of experiment would be that of antibody excess, so that no free antigen would exist. Accordingly these experiments were carried out with an antigen-antibody ratio of about 1:28 (1:40,000 antigen dilution) as shown in the following typical experiment.

0.2 mg. of enzyme in 0.2 ml. volume was added to 5 ml. of antiserum. This mixture was allowed to react in a 37°C. water bath for 1 hour, then placed in the refrigerator for 48 hours, centrifuged, and the precipitate washed once with chilled 0.85 per cent NaCl solution. The precipitate was made to 1 ml. with 0.85 per cent NaCl

solution and then 1 ml solution (10 mg) of *d*-ribonucleic acid was added and the mixture stirred well to insure a uniform suspension. After the 30 minutes' time allowed for this reaction the mixture was centrifuged and the 0.5 ml of clear supernatant was used for a phosphorus determination. An equal volume of uranium acetate reagent<sup>1</sup> (exactly 1.5 ml) was added to the 1.5 ml of the clear supernatant remaining and the mixture allowed to stand for 20 minutes. The precipitate which contained the unhydrolyzed nucleic acid was then removed by centrifugation and the amount of phosphorus was determined in 2 ml of the supernatant which contained the hydrolyzed nucleic acid. A control was run, lacking only the antiserum.

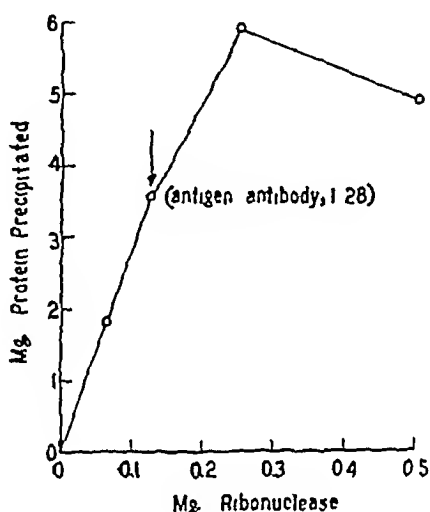


FIG 1

The results of the two experiments were control—43 per cent hydrolysis<sup>2</sup> of the nucleic acid, with antiserum—30 per cent hydrolysis of the nucleic acid

$$\frac{43 - 30}{45} \times 100 = 30 \text{ per cent inhibition}$$

control—45 per cent hydrolysis of nucleic acid, with antiserum—35 per cent hydrolysis of nucleic acid

$$\frac{45 - 35}{45} \times 100 = 22 \text{ per cent inhibition}$$

The above results were obtained using a precipitate from an antigen-antibody combination which had been allowed to react for 48 hours

<sup>1</sup> 0.25 per cent uranium acetate in 2.5 per cent trichloroacetic acid (3)

<sup>2</sup> These experiments were carried out at pH 7.2 which is not the pH of optimal enzyme activity. A more alkaline pH was not used in order to avoid the dissociation of antigen-antibody combination.

Other similar experiments carried out with only 24 hour antigen antibody incubation, showed inhibition ranging from 10 to 15 per cent

#### DISCUSSION

The results of the present study show that crystalline *d* ribonuclease is antigenic. This enzyme stimulates the formation of antibodies when injected into rabbits, using different methods of immunization, and reacts with the homologous antisera in very high dilutions of antigen. Different preparations of the crystalline *d* ribonuclease appear to exhibit different behavior in stimulating this antibody formation. Some antisera prepared against different enzyme preparations, when tested against these enzymes in the precipitation test showed a prozone which at times went up as high as 1:100,000 antigen dilution. Other antisera, however, showed no prozone at all. Any one antiserum gave identical precipitation against all of the purified enzyme preparations. For instance, antiserum prepared against the five times crystallized enzyme gave the same precipitation titer when tested against the five times and eight times crystallized enzymes (see Table II).

Numerous papers have appeared of the inhibition effect of antisera on homologous enzyme antigens. Kirk and Sumner showed that antiserum against urease inhibited urease activity (8), Lüers and Albrecht showed that anti-amylase serum inhibited amylase activity (9), and Macfarlane and Knight found that lecithinase activity of *Clostridium welchii* (*Clostridium perfringens*) toxin was inhibited by antitoxin (10).

Our attempts to determine whether the ribonuclease activity could be inhibited by its homologous antisera were met with certain technical difficulties. The quantitative determination of the hydrolyzed nucleic acid (P determination) in systems containing the enzyme and the immune serum was not practicable because of the large amount of protein present. Methods employed to eliminate the protein by the use of precipitating agents would also precipitate the nucleic acid (4). Therefore the inhibitory effect of the specific antiserum was determined by using the washed antigen-antibody precipitate as the enzyme material. In the reaction mixture containing the nucleic acid this precipitate was finely suspended, and could be removed easily at the end of the reaction by centrifugation. The results showed that the antibody inhibited the enzyme activity from 10 to 30 per cent.

The question may be raised as to whether this reduction of the enzyme activity is really due to the blocking of active groups on the enzyme by combination with antibody or due to the physical inability of the large molecular weight nucleic acid to penetrate the suspended particles and thus come into contact with the active enzyme groups. We are unable to answer this question. However, Lüers and Albrecht observed the inhibition of amylase by its homologous antibody using starch, which is a high molecular weight substance, as substrate

In other similar studies referred to above, this question did not arise since the substrates such as urea and lecithin are of relatively small molecular weight and can readily penetrate into the suspended particles of the antigen-antibody combination.

#### SUMMARY

The enzyme *d*-ribonuclease is antigenic. Antisera, prepared by three different methods, reacted against antigen dilutions up to one million.

Apparently the homologous antiserum, when combined with the *d*-ribonuclease inhibited the activity of the enzyme from 10 to 30 per cent.

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# THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

## IV THE RELATIVE MERITS OF THE HOMOGENEOUS PHASE THEORY AND THE MICELLAR-STRUCTURAL THEORY AS APPLIED TO THE DRIED COLLODION MEMBRANE

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### I

The structure of the dried collodion membrane has in the past been the subject of an extensive controversy. A great variety of arguments<sup>1-4</sup> has been put forward to substantiate the one or the other of the two major conflicting theories, which are referred to frequently as the "solubility theory" and the "pore theory" respectively. Instead of these terms, which are rather suggestive of special mechanisms, we intend to use in this paper the more general, less suggestive terms, "homogeneous phase theory" and "structural" or preferably "micellar structural theory" respectively.

It is not the plan of this communication to collect and re-evaluate the arguments used in this controversy. In our opinion an inadequate terminology has contributed considerably to the blurred state of the problem. However, it is not the purpose of this paper to discuss this point.

From the experimental point of view we should like to make the following remark concerning the physical aspects of the problem. It seems advisable to consider more closely the possibility of the existence of different mechanisms of permeation according to the nature of the substances which permeate a

<sup>1</sup> See, e.g. Beutner R., *Die Entstehung elektrischer Ströme in lebenden Geweben* Stuttgart, Ferdinand Enke, 1920. Beutner, R., *Physical chemistry of living tissues and life processes as studied by artificial imitation of their single phases*, Baltimore, The Williams & Wilkins Co., 1933. Bentner, R., Caplan, M. and Loehr, W. M., *J Biol Chem*, 1933, 101, 391. Beutner R. *Arch Zellforsch* 1934, 15, 217.

<sup>2</sup> Collander, R. *Kolloidchem Beihfte*, 1924, 19, 72, 1925, 20, 273. *Societas Scientiarum Fennica Commentationes Biologicae* II 6 Helsingfors, 1926.

<sup>3</sup> E.g. Michaelis, L., and Perlzweig, W. A. *J Gen Physiol*, 1926-27, 10, 575, Michaelis, L., McEllsworth, R., and Weech, A. A., *J Gen Physiol*, 1926-27, 10, 671. Michaelis, L., Weech, A. A., and Yamatori, A., *J Gen Physiol*, 1926-27, 10, 685. Michaelis, L., *Bull Nat Research Council No 69* 1929, *Kolloid Z*, 1933, 62, 2, and other publications.

<sup>4</sup> E.g., Northrop J. H., *J Gen Physiol* 1928, 11, 233, 1929, 12, 435.

<sup>5</sup> E.g. Wilbrandt, W., *J Gen Physiol*, 1935, 18, 933.

membrane Closer consideration should also be given to the state of the latter, it may be that collodion membranes in the dry state behave differently (for their permeability for gases) than those in the wet state Also the affinity of the permeating substance for the membrane material can conceivably play a much more important and characteristic rôle than generally assumed Strong inorganic electrolytes, for example, may easily follow pathways essentially different from those which are available to compounds which in higher concentration act as swelling agents, solvents, etc Conclusions reached on the basis of experiments with one kind of substances should not be extended without further proof to other groups It is even conceivable that one theory may fit one set of circumstances, whereas the other theory may fit a different situation

Before we begin to discuss the special approach to the general problem which is made in this paper, we should like to mention two independent sets of observations which, though pertinent, have been largely neglected in the literature on membranes

It is well known that dried collodion membranes cast from the same solution may show widely varying concentration potentials even if they are cast under conditions which are kept constant as far as experimentally possible This effect is particularly conspicuous with membranes made from electrochemically inactive preparations, here the variations in concentration potential across different membrane specimens are frequently more than 100 per cent,<sup>5</sup> the potentials across the individual membranes being very constant and easily reproducible over long periods (See Tables 1a and 1b) The homogeneous phase theory could not conceivably account for this variability of the concentration potential, in fact, it actually seems to be incompatible with it All specimens of a homogeneous phase should have the same properties whatever their past, the more so if prepared under identical conditions A structural theory, however, could easily accommodate the observed facts Differences in aggregation and orientation of molecules and of micelles could easily be accounted for by uncontrollable variations in the preparation of the individual membranes Since any structural theory necessarily assumes that orientation and aggregation are the factors which determine the structure of membranes and thus their behavior, such a theory seems greatly favored by the above mentioned variations in concentration potential

Furthermore we feel that inadequate use has been made of the evidence in favor of the micellar-structural theory which is contained in the literature on x-ray studies of nitrocellulose This literature demonstrates definitely the occurrence of crystalline micelles in nitrocellulose films, the sharpness of the interferences varying greatly with different preparations It would be attractive and profitable to discuss the extent to which x-ray evidence indicates either a truly micellar structure or merely the presence of micelles in an amor-

pious matrix. However, we shall limit our remarks to the statement that the micellar-structural theory receives strong support from the x ray literature

## II

The present work is an attempt to determine which of the two theories—the homogeneous phase theory or the micellar structural theory—allows us to interpret the observed behavior of dried collodion membranes with solutions of strong electrolytes

The following line of reasoning serves as a basis for our present experiments. A number of dried collodion membranes are prepared under standardized conditions from some electrochemically inactive brand of collodion (state I). The characteristic concentration potential across a representative group of these membranes is determined. Next, the membranes are “activated” by oxidation (state II) as described previously<sup>6</sup> and the concentration potential of a representative number of these membranes is determined. The remaining majority of the membranes in state II are dissolved in the same solvent mixture as used originally. Dried collodion membranes are prepared from this solution (state III), the concentration potentials across these membranes are measured, and the results obtained in states I, II, and III are compared.

The homogeneous phase theory would predict that the properties of the membranes in state II and state III are substantially identical since the properties of an interphase which behaves like a homogeneous phase should be independent of its history. No rearrangement of the molecules, brought about by any means, should be able to alter the properties of membranes prepared from the same material.

The micellar-structural theory would predict a distinct difference between state II and state III. According to this view, the charges which are caused by chemical reactions in the membranes, or by the addition of third substances, are substantially confined to certain more accessible structural elements,  $\pm e$ , the surfaces of the particles which constitute the membrane. If the membrane is dissolved, the elements which are the cause of the characteristic properties of state II are mixed with the whole mass of the membrane material. If a new membrane is now prepared from this solution, the concentration of the active groups at the accessible points is necessarily reduced as compared with state II.

The foregoing discussion is based on the tacit assumption that the interstices which govern the behavior of the membrane are larger than the minimal possible molecular interstices. Such interstices may arise due to an irregular arrangement of molecules without the formation of distinct micelles or in the formation of micelles and their arrangement. In either case the effective interstices would be of micellar as opposed to molecular magnitude, and either

<sup>6</sup> Sollner, K. Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, 25, 7.



case would fulfill the necessary condition for the above considerations. We designate them as having a "micellar-structural" constitution, as opposed to the molecular-structural constitution of a crystal or liquid, the emphasis, as expressed in our terminology, is more on the nature of the interstices than on the structure-forming physical elements. That the formation of micelles, at least in a rudimentary manner, is *a priori* the more likely situation with the fibrous nitrocellulose molecules is here without particular importance. The homogeneous phase theory, of course, must necessarily assume a molecular-structural constitution of a crystalline or glasslike liquid character with true equivalence, or at least statistical equivalence, for all actual or virtual interstices. The behavior to be expected from a membrane having such a molecular-structural constitution was pointed out in a preceding paragraph.

### III

A necessary prerequisite of the experiment, of course, is that the activation is not too thorough. If the number of active groups which are introduced in state II is so great that too many of them come to lie in the critical spots in state III, the results may become less pronounced. In order to demonstrate the anticipated effect most clearly, the activation should be stopped just at the time when maximal or nearly maximal concentration potentials are obtained. A more prolonged oxidation would necessarily lead to a gradual diminution in the expected effect. One further has to ascertain that a significant loss of active material does not occur at any state of the whole experiment, otherwise its purposeful evaluation is impossible.

Below we report two series of experiments carried out according to the plan outlined. In the one series (series *a*), the degree of oxidation applied is about the minimal which yields membranes of high activity, series *b* was subjected to a much stronger oxidation.

For the preparation of the original membranes (state I), Mallinckrodt "Parlodion" purified by boiling 7 hours with 90 per cent alcohol<sup>7</sup> was used, this purified material has been shown to be the least active of the available collodion preparations. 5 gm per 100 ml were dissolved in a mixture of 25 per cent absolute alcohol and 75 per cent absolute ether, this mixture being adhered to throughout all the work reported in this paper.

For each of the two experimental series, 70 bag-shaped membranes were cast by hand in 22 × 85 mm pyrex test tubes and allowed to dry in the air at room temperature for 48 hours. After 48 hours the membranes are perfectly dry and can be removed with water from the test tubes, the uneven, frequently heavy rims of the membranes are cut off. The average thickness of these membranes is about 10  $\mu$ , as calculated from area and weight, the specific gravity of collodion being 1.6. Appreciably thinner and thicker spots are found on

<sup>7</sup> Sollner, K., Carr, C. W., and Abrams, I., *J. Gen. Physiol.*, 1942, 25, 411.

each individual membrane. Out of each group of 70 membranes (state I), every seventh was picked to obtain a representative group of ten membranes for the determination of the characteristic concentration potentials. For these measurements the membranes were fitted loosely over glass rings, held in wooden test tube clamps, and filled about two-thirds with 0.1 molar potassium chloride solution. They were immersed to the same level in 0.01 molar potassium chloride solution (temperature about 25°C). Potential measurements were made about 5 minutes after the membranes were interposed between the potassium chloride solutions and repeated afterwards to ascertain

TABLE I

*The Concentration Potential 0.1 M KCl/0.01 M KCl across Dried Collodion Membranes Prepared from Purified Parlodion\* (State I) Potentials in Millivolts*

(Series a)			(Series b)		
Membrane	Concentration potential		Membrane	Concentration potential	
	5 min	2 days		5 min	2 days
	mv	mv		mv	mv
1	35	36	71	26	(8)*
8	28	26	78	34	35
15	28	27	85	31	33
22	27	25	92	30	35
29	37	38	99	29	29
36	32	33	106	26	28
43	20	21	113	37	37
50	13	15	120	36	36
57	33	33	127	29	28
64	22	22	134	26	27
Mean	27.5	27.6	Mean	30.4	32.0

\* This membrane was damaged when the potential measurement was made. The value in parenthesis is not considered in the calculation of the mean.

the stability of the potential. The 5 minute value was found to be the final stable potential in nearly all cases. The accuracy of these determinations is  $\pm 1$  mv. On account of the high ohmic resistance of the membranes in state I, it was convenient to use the volt scale of a commercial (Leeds and Northrup) glass electrode set, in spite of its limited ( $\pm 1$  mv) accuracy.

The two series of ten membranes each in state I were kept in distilled water (thymol being present as a preservative) and the concentration potential measured again after 2 days. The potential measurements performed with the membranes in state I are summarized in Tables Ia and Ib, the mean values for the two series are 27.5 and 30.4 mv respectively, the difference being statistically insignificant.

The remaining two sets of 60 membranes each were oxidized under the following conditions, series *a*, 3 hours oxidation time in a 0.05 molar<sup>8</sup> sodium hypobromite solution at pH 6, series *b*, 1 hour in molar<sup>8</sup> hypobromite solution at the same pH

TABLE II

*The Concentration Potential 0.1 M KCl/0.01 M KCl across Dried Collodion Membranes Activated by Oxidation with Sodium Hypobromite Solution (State II), Potentials in Millivolts*

<sup>a</sup> (Series <i>a</i> , 3 hrs oxidation with 0.05 M potassium hypobromite solution)			<sup>b</sup> (Series <i>b</i> , 1 hr oxidation with 1.0 M potassium hypobromite solution)		
Membrane	Concentration potential		Membrane	Concentration potential	
	5 min	2 days		5 min	2 days
	<i>mv</i>	<i>mv</i>		<i>mv</i>	<i>mv</i>
2	50	(0)*	72	52	52
3	50	50	73	52	49
9	50	(0)*	79	50	48
10	50	50	80	52	51
16	50	48	86	51	50
17	50	50	87	50	49
23	50	50	93	53	51
24	50	49	94	53	51
30	50	47	100	50	50
31	50	50	101	52	51
37	50	50	107	53	51
38	49	45	108	52	50
44	50	(0)*	114	51	50
45	50	50	115	52	50
51	50	50	121	52	51
52	50	50	122	50	49
58	50	(0)*	128	51	51
59	50	48	129	51	50
65	49	50	135	50	50
66	50	50	136	50	51
Mean	49.9	49.2	Mean	51.3	50.2

\* See footnote to Table I

After oxidation the membranes were washed thoroughly with distilled water with frequent changes of water. Out of each of the two groups of 60 membranes, 20 membranes were picked as indicated in Table II. These membranes were considered as representative of the two groups, the characteristic con-

<sup>8</sup> The term "molar" refers here to the concentration of the sodium hydroxide solution from which the hypobromite solution is prepared by the addition of bromine (Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, 25, 7)

centration potentials obtained with them after about 5 minutes (as before) are listed in Table II *a* and II *b*. The tables also give the mean potential values, 49.9 mv for the less thoroughly oxidized series and 51.3 mv for the more strongly oxidized membranes.

TABLE III

*The Concentration Potential 0.1 M KCl/0.01 M KCl across Dried Collodion Membranes Prepared from Redissolved Activated Membranes (State III) Potentials in Millivolts*

<sup>a</sup> (Series a)			<sup>b</sup> (Series b)		
Membrane	Concentration potential		Membrane	Concentration potential	
	5 min.	2 days		5 min.	2 days
	mv	mv		mv	mv
a	32	33	A	41	40
b	33	33	B	38	36
c	20	22	C	49	48
d	26	25	D	34	34
e	22	22	E	50	48
f	28	28	F	49	(31)*
g	35	41	G	18	20
h	13	13	H	45	45
i	28	29	I	22	22
j	28	28	J	40	40
k	16	16	K	32	36
l	28	29	L	40	41
m	28	30	M	39	39
			N	12	13
			O	44	44
			P	47	48
			Q	40	(31)*
			R	20	20
			S	47	47
			T	47	48
Mean	26.0	26.8	Mean	37.7	37.2

\* See footnote to Table I.

After the potential measurements were made these membranes were washed in distilled water and kept for 2 days in distilled water (with thymol). The concentration potentials were measured again after this time. No significant amount of material which causes the electro-chemical behavior of the membranes has been washed out during this 2 day period.

The 40 remaining membranes in state II of each of the two series were air dried and dissolved in the standard ether-alcohol mixture. Membranes were cast from the resulting solution and dried (state III) as described before, each

solution allowing the preparation of only a limited number of membranes. These membranes in state III were removed from the glass tubes and the concentration potentials were measured after about 5 minutes contact with the electrolyte solutions. The measurements were repeated after 2 days, as before, to determine whether any change in activity occurs. This, however, was not the case. The thickness of these membranes averaged about  $10\ \mu$  as before. Table III *a* and III *b* give the potential measurements of the membranes in state III pertaining to the two series of experiments. A few obviously damaged membranes giving zero or very low potentials are not recorded in the table. The mean values in state III are 26.0 and 37.7 mv respectively.

It is hardly necessary to point out that the moderate oxidation in series *a* gives the more clear cut results, as compared with series *b* in which the oxidation was more thorough. We may add that a third series of analogous experiments was carried out in which the oxidation was still more thorough than in series *b*. In agreement with expectation, the average concentration potential in state III was in this case rather high, about 49 mv.

It may be worth-while to record the fact that the membranes in state III behave like normal dried collodion membranes prepared from inactive collodion. The membranes of state III of series *a* after thorough oxidation gave in this new state (state IV) high characteristic concentration potentials, the mean value being slightly higher than 51 mv.

#### IV

The experimental results recorded in Tables I, II, and III are so unequivocal, as to necessitate hardly any discussion. The concentration potentials obtained in state III are much lower than in state II, the concentration potential in state III in our series *a* being actually identical (within experimental accuracy) with that of state I. If we recall the previously outlined predictions of the "homogeneous phase" and the "micellar-structural" theories, we see immediately that the experimental results agree excellently with the predictions of the micellar-structural theory, they are incompatible with the predictions and therefore with the basic assumptions of the homogeneous phase theory.

Micellar and not molecular interstices are the factors governing the behavior of dried collodion membranes—at least in the case of strong inorganic electrolytes. This, of course, does not imply that molecular interstices do not exist, they must necessarily exist. It means only that their existence is not an important factor in the characteristic membrane behavior, this is true even if it should turn out that the total volume of the molecular interstices exceeds the total volume of the micellar interstices. There is no indication that all the micellar interstices are of the same size or that they are equally accessible,

rather, non-equivalence in both respects has to be expected as indicated previously<sup>7</sup> for the case of the porous collodion membrane. Some direct experimental evidence as to the non-equivalence of the micellar interstices is already contained in the literature.<sup>8</sup> It seems probable that a mere fraction of the micellar interstices, the readily accessible ones, determine the characteristic membrane behavior. The obvious methods of attacking these and similar problems involve quantitative studies of the rates of water and electrolyte uptake by the membranes and of the final equilibrium. These studies must be combined with conductance and concentration potential measurements. However, all such studies depend upon information upon the water uptake and swelling of the dried collodion membrane. Such studies are under way at this time.

#### SUMMARY

1 Experiments were carried out to decide whether a homogeneous phase (solubility) theory or a micellar structural theory more adequately describes the behavior of dried collodion membranes with solutions of strong electrolytes.

2 A number of dried collodion membranes were prepared from an electrochemically inactive collodion preparation (state I), the characteristic concentration potentials across them were low, about 30 mv. The membranes were activated by oxidation (state II) to give maximum or nearly maximum concentration potentials (about 50 mv). The oxidized membranes are dried, dissolved in alcohol-ether, and a new set of dry collodion membranes prepared from this solution (state III). The concentration potentials across these membranes are low.

3 Since the properties of a homogeneous phase should not be influenced by a rearrangement of its constituent particles, the experimental results do not support a homogeneous phase (solubility) theory, but they agree with the predictions of the micellar-structural theory. The characteristic behavior of dried collodion membranes in solutions of strong inorganic electrolytes is therefore due to the micellar character of its interstices.

<sup>8</sup> Green, A. A., Weech, A. A. and Michaelis, L., *J. Gen. Physiol.* 1929, 12, 473



# THE REGENERATION OF VISUAL PURPLE IN THE LIVING ANIMAL

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## I

### *Vision and Visual Purple*

When an animal, which has been in the light, is placed in the dark, its sensitivity to light increases. Measured as the light intensity necessary to elicit a minimal visual response, these changes yield the familiar data of dark adaptation (Aubert, 1865, Piper, 1903, Hecht, 1937).

For many years it has been known that during dark adaptation the vertebrate retina shows changes in concentration of the photosensitive rod pigment visual purple (Kühne, 1878, Gatti, 1897, Fridericia and Holm, 1925, Tansley, 1931, Zewi, 1939). More recently the chemical behavior of this substance has become associated with certain carotenoids (Wald, 1938).

The present research was undertaken to discover the course of the concentration changes shown by visual purple in the intact animal, to relate this information to the data of dark adaptation, and to elucidate the behavior of the carotenoid pigments. As a working hypothesis it is assumed that dark adaptation depends on the accumulation of visual purple in the retina. It is proposed, therefore, to use those conditions during regeneration of visual purple which are known to modify dark adaptation in specific ways and to see whether similar effects are produced on the course of visual purple accumulation.

## II

### *Dark Adaptation and Visual Purple*

Due to the work of Hecht (1921) and of Kohlrausch (1922) it is known for the human eye that the two receptor systems—rods and cones, both enter into normal dark adaptation. Following daylight illuminations, human dark adaptation proceeds in two stages. The first is rapid and is practically complete in 3 or 4 minutes; it records mainly cone function. The second is delayed, slow, and takes about 25 minutes for completion; it records rod function.

There are many factors which control the extent, speed, and duration of the two parts of the course of dark adaptation. The most striking are the intensity and duration of the light preceding the beginning of dark adaptation. Following high intensity light adaptation, the threshold falls in two steps. Decreasing the intensity of light adaptation diminishes the extent of the first section and shortens the time at which the transition from cone to rod function



occurs Following the lowest intensities of light adaptation, cone function does not appear at all, and only the secondary rod adaptation is evident (Johannsen, 1934, Winsor and Clark, 1936, Hecht, Haig and Chase, 1937) In other words, the portion of dark adaptation mediated by the rods is delayed when the eye is light adapted to high intensities, and begins without delay following low intensity light adaptation

A similar effect on the course of dark adaptation is produced by varying the duration of light adaptation Müller (1931) found that following a short period of light adaptation only the secondary rod dark adaptation appears With increasing time of light adaptation, the primary cone adaptation appears and increases in duration until it occupies the first 5 minutes of the dark adaptation process, while the second, rod portion, is delayed for greater periods These results were later confirmed by Wald and Clark (1937)

Among the many animals whose dark adaptation has been measured, the frog is of particular interest here It too possesses rods and cones and may be expected to show a two-stage adaptation This has been found by Riggs (1936-37) who, following the method of Hartline (1930), used as an index the B wave of the retinal potential elicited by a brief illumination of the intact dark adapting eye This method is comparable with the procedure used on the human eye In the latter the data represent the light intensity required to produce a constant visual effect at various times in the dark The procedure of Hartline and Riggs is to determine the intensity of light which produces a constant physiological effect, in this case a given retinal potential, at different times in the dark The course of adaptation divides into two sections, the first corresponding to cone function, and the second to rod function The rods do not mediate the function until the cone portion of the data has reached completion approximately 10 minutes after the onset of dark adaptation The rod function reaches a threshold about 1 hour after the start of dark adaptation

The results of Granit, Holmberg, and Zewi (1938) and of Granit, Munsterhjelm, and Zewi (1939) on frog dark adaptation seem at first sight completely at variance with the human data and those of Riggs Instead of a rapid increase in sensitivity as found by Riggs, Granit and his coworkers report delays of as long as 1 hour before a rise in sensitivity begins Moreover, in spite of the fact that the frog retina contains both rods and cones and that the adapting light is of sufficient intensity to show their separate effects, the data are continuous without any indication of a double function

This apparent discrepancy between the work of Granit and of Riggs is probably due to the method used by Granit in which the *size* of the retinal potential evoked by a measuring light of constant intensity is considered the measure of sensitivity This method is not comparable either to the accepted practice in direct visual measurements or in retinal potential measurements of

visual function (*cf* Chaffee, Bovie, and Hampson, 1923, Hartline, 1930), in which there is always measured the variation in intensity required to produce a constant physiological or electrical effect. It is quite likely that the reason Granit found only a single and very delayed function in frog dark adaptation is that the *magnitude* of the retinal potential does not correspond to visual sensitivity as measured in the usual ways.

Considering the relationship of visual purple to vision, it would be desirable to know how these changes in the sensitivity of the eye depend upon variations in the concentration of visual purple. For conditions at the stationary state, a fairly successful description of many visual functions has been made upon the basis of simple photochemical theory derived from consideration of the equilibrium concentration of a photosensitive material like visual purple (Hecht, 1938). For kinetic measurements like dark adaptation, however, there exists no adequate description either in terms of theory or in terms of actual measurements of visual purple concentration.

Kühne (1878) showed that both the intact retina and solutions of visual purple, when returned to the dark, would regenerate visual purple which had been bleached by light. Hecht, Chase, Shlaer, and Haig (1936) confirmed the regeneration of visual purple in solution. Gatti (1897), and later Fridericia and Holm (1925), by comparisons of the retina with a series of standardized color charts, investigated the accumulation of visual purple in the dark adapting retina. More quantitative measurements were made by Tansley (1931), who studied spectrophotometrically digitonin extracts of dark adapting rat retinas. She found a gradual increase in visual purple concentration during dark adaptation, and the speed of the process was retarded by lack of vitamin A in the diet. Zewi's (1939) work on the regeneration of visual purple in the intact frog showed among other things that the pigment begins to increase in concentration as soon as the animal is placed in the dark except following short periods of light adaptation or at low temperature.

In addition to the changes in visual purple during dark adaptation, there are also changes in the carotenoids of the retina. Wald (1935, 1936, 1937, 1938) demonstrated the rôle of these carotenoids in the visual cycle. Excised retinas, when freshly bleached, contain a carotenoid which Wald called retinene. If the retinas then remain either in the light or in the dark, the retinene disappears and in its place vitamin A appears. This change is independent of light and is a thermal process. According to Wald, visual purple is regenerated along two paths. One is directly from the photoproducts acting with other materials which are at hand in the retina. The other is through the conversion of retinene into vitamin A, and the combination of vitamin A with a specific protein to form visual purple.

Much information, however, is still needed to establish the quantitative

interrelations among dark adaptation, visual purple regeneration, and carotenoids. It is hoped that the following measurements will supply some of this information.

### III

#### *Apparatus*

The apparatus is essentially a device for illuminating the frog so that the eye will become evenly adapted. Two such arrangements were used.

The first consists of a metal sphere *S* 12 inches in diameter (Fig 1). At equal intervals on the inside of the sphere, seven automobile headlight lamps of 6-8 volts

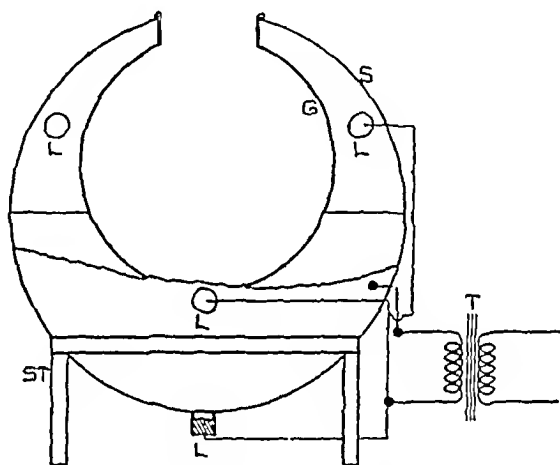


FIG 1 First apparatus for light adaptation. The metal sphere *S* has on its inside a series of lights *L*, which illuminate evenly the opal glass globe. The frogs are in the globe which is kept at constant temperature by circulating water through it.

are mounted, and the whole group is operated at 170 watts through a transformer *T*. The inside of the sphere is painted flat white to insure a diffuse reflection. A spherical opal glass globe *G* is suspended inside the sphere and so arranged that it is evenly illuminated.

The temperature inside the globe is regulated by a continuous flow of cooling water entering on one side of the globe and leaving by suction on the opposite side.

For light adaptation the frogs are firmly fixed upon a board and placed inside the globe in the circulating water.

High intensities could not be obtained with this apparatus because the sphere was too small to permit mounting a sufficient number of lamps. Moreover, at high intensities it was impossible to prevent the temperature of the globe from rising.

The second apparatus overcame these difficulties. It consists of a conical, aluminum-painted reflector *R*, containing a 1000 watt lamp *L* (Fig 2). The heat generated by the lamp is carried off through vents. The frog is placed in a white enamel

receptacle *A*, which is carefully fitted with a cover of opal glass *O*, over which is a pyrex glass *H*. The receptacle is then securely inserted underneath the conical reflector in such a way that the opal glass plate, as well as the walls of the receptacle, are evenly illuminated. The animals are cooled during light adaptation by a continuous flow of water which enters at the bottom of the receptacle *I* and leaves at the top *X*.

The intensity of the illumination is determined with a Macbeth illuminometer, and is checked during the course of the experiments.

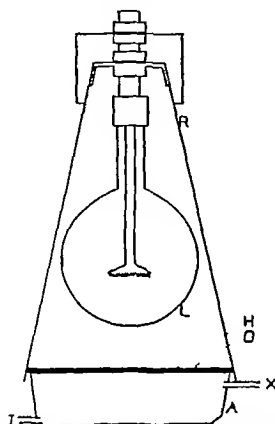


FIG 2 Second apparatus for light adaptation. Light from a 1000 watt lamp *L* is reflected by the metal housing *R* on to the opal glass *O* through the heat-absorbing glass *H*. The frogs are in the white enamelled receptacle *A* and are kept at a fixed temperature by water entering at *I* and leaving at *X*.

For dark adaptation, the animals are placed in a water bath kept entirely in the dark and maintained at constant temperature.

#### IV

#### *Procedure*

(a) *Manipulations*—The frogs were all *Rana pipiens*, obtained from Vermont and stored for only a short period in the laboratory before use. They were light adapted two at a time. The cooling water was started and the light turned on for periods of

5, 10, or 20 minutes After the light was turned off, the animals were immediately put into the constant temperature bath for dark adaptation The subsequent manipulations were carried out in dim red light from which the frogs and retinas were shielded as much as possible

After dark adapting for specific periods, the two frogs were removed from the bath and immediately beheaded The eyes were removed and dropped into water containing cracked ice The eyes were then immediately sectioned behind the iris, and the retina and pigment layer removed into buffer solution of pH 9.6 which was kept at 0°C The procedure after this depended on the measurements

(b) *Visual Purple Determination*—As extractive, 4 cc. of a 4 per cent solution of sodium desoxycholate in Clark and Lubs boric acid-KCl buffer mixture (Clark, 1928) of pH 9.6 were used This choice of extractive rests on the fact that the regeneration of visual purple in solution (Hecht, Chase, Schlaer, and Haig, 1936) occurs neither in sodium desoxycholate (Chase and Smith, 1939) nor at a pH above 9.0 The extractive containing the four retinas was kept at 0°C until the second set of retinas was similarly prepared and added to the solution The suspension was stirred with a glass rod to break up the retinas, and then placed in a bath at 25°C for 1 hour during which it was frequently agitated

After extraction, the solution was centrifuged for 60 to 90 minutes This operation was carried out at 6°C in order to prevent an undue temperature rise due to the centrifugation, since visual purple is decomposed at high temperature (Kühne, 1878, Lythgoe and Quilliam, 1938) The clear supernatant liquid was carefully removed with a pipette, and transferred to small test tubes which were stored at 6°C

The concentration of visual purple was determined by measuring the transmission of a 1 cm layer of the solution at 500 m $\mu$  with Schlaer's photoelectric spectrophotometer (Schlaer, 1938) within 20 hours of extraction Without removing it from the spectrophotometer, the solution was completely bleached by a 10 minute exposure to the light of a 250 watt projection lamp placed 6 inches from the solution and separated from it by a filter of heat-absorbing glass The rise in temperature was seldom more than 1.5°C After bleaching, the solution was permitted to return to room temperature and the transmission at 500 m $\mu$  again determined The density of visual purple was then computed from the difference in transmission between the bleached and unbleached solution

(c) *Carotenoids*—For carotenoid estimation the retinas were separated from the pigment layer Eight retinas were extracted together three times with petroleum ether, each extract employing 2 cc. of petroleum ether The successive extracts were combined and evaporated to dryness at reduced pressure The residue was then taken up in 2 cc. of anhydrous chloroform To test for the presence of vitamin A and other carotenoids, the Carr-Price (1926) reaction with antimony chloride was used The chloroform extract was placed in an absorption cell, and 5 cc. of a saturated antimony chloride solution in chloroform were added The optical density of the resulting blue solution was determined through a thickness of 1 cm. of solution at 612 m $\mu$  and 664 m $\mu$ , corresponding to the absorption maxima of the SbCl<sub>3</sub> compounds of vitamin A and retinene respectively Since the blue color fades rapidly, successive measurements were made, and from the plot of optical density against time of measurement the density at the moment of mixing was determined by extrapolation

## V

*Visual Purple Regeneration*

(a) *Adaptation at 25° C*—Two series of experiments were performed at 25° C. In one, light adaptation was to 1700 millilamberts, in the other to 9500 millilamberts, both for 10 minutes, and both followed by dark adaptation at 25° C.

The results are presented in Table I, where each density is the average of the individual measurements shown in Fig. 3. Each individual point represents an extract of eight retinas. The curves are smoothly drawn as close to the averages as possible. They are nearly similar in shape and in position, and in both cases the initial concentration of visual purple is not zero, but a finite value.

The significant difference between the data obtained at the two intensities is that following 1700 millilambert light adaptation, the regeneration of visual purple begins immediately, while at the higher intensity the onset of regeneration is delayed. This delay at 25° C lasts for about 10 minutes before a rapid rise in the concentration of visual purple begins. After regeneration starts, it proceeds rapidly and reaches a plateau approximately 75 minutes after the frogs have been placed in the dark.

These data may be compared with the behavior of the rods in adaptation. Hecht, Haig, and Chase (1937) found for human vision that recovery of rod sensitivity in the dark begins without any delay following low intensity light adaptation, while at high intensity light adaptations, rod function is increasingly delayed. The data in Fig. 3 show a similar difference between low and high intensity, though the actual intensities of light adaptation differ.

The data for the higher intensity light adaptation agree with the results of Riggs (1936-37) for the intact eye of *Rana pipiens*, which show a delay of about 10 minutes in the appearance of rod dark adaptation. It may thus be that this delay in the assumption by the rods of the threshold function in frogs during dark adaptation is due to the lag in the formation of visual purple.

The data of Zewi upon the regeneration of visual purple differ considerably from those presented here. At 25° C and at comparable intensities of light adaptation, his data show no suggestion of delay in the recovery of visual purple. His data do not correspond with those of Riggs, which are typical of all dark adaptation data, or with the data of Granit which show very long delays in the recovery of sensitivity.

(b) *Adaptation at 15° C*—The experiments were repeated at 15° C. In this way they duplicate the conditions of Riggs' measurements on the intact eye so that comparisons of the visual purple regeneration curves with the curve describing the recovery of sensitivity in the intact eye can be made.

The data are given in Table II where each density is the average of the

individual measurements shown in Fig 4 Each individual point represents an extract of eight retinas The curves are drawn through the averages as before

The measurements show that there is little difference between the regeneration of visual purple at the two intensities The concentration of visual purple increases more rapidly after lower light adaptation, and reaches the maximum slightly earlier However, it is evident that a decrease in temperature of  $10^{\circ}\text{C}$  has delayed the process of regeneration to such an extent that the acceleration expected by lower light adaptation has been considerably diminished

TABLE I

Changes in concentration of visual purple at  $25^{\circ}\text{C}$  in retinas of frogs after 10 minute light adaptation Series I, light adaptation at 1700 millilamberts, Series II, 9500 millilamberts

Time in dark <i>min</i>	Photometric density	
	Series I	Series II
0	0 012	0 015
5	0 014	0 014
10	0 016	0 016
15	0 022	0 027
20	0 028	0 042
25	0 037	0 057
30	0 046	0 070
35	0 051	0 072
40	0 054	0 079
45	0 069	0 089
50	0 075	
60	0 086	0 128
75	0 106	0 135
90	0 108	0 133
120	0 110	0 135

The initial delay in visual purple accumulation appears at both intensities of light adaptation The limiting factor in this case is apparently the temperature of dark adaptation rather than the intensity of light adaptation

These data again indicate that following its decomposition by light of sufficient intensity, visual purple does not begin to regenerate immediately There is an initial period during which little or no visual purple forms, which lasts approximately 10 minutes, and is followed by a rapid rise in concentration

The curves are sigmoid in shape, the only differences between them being the time elapsing before the rapid upward portion starts It is also to be noted that there is a slight difference in the total period elapsing between the cessation of light adaptation and the time at which the final level of visual purple is reached

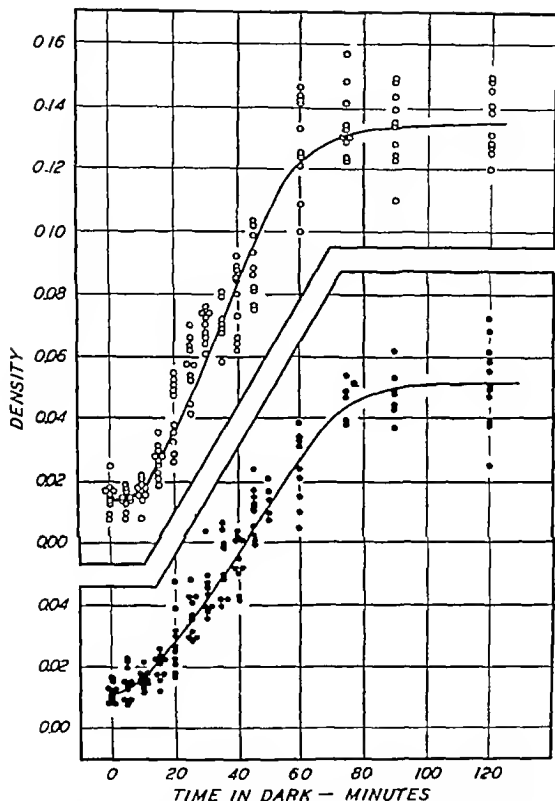


FIG 3 Regeneration of visual purple in the intact eye of the frog at 25°C. The concentration is measured as density at 500 m $\mu$ . Open circles, visual purple concentration following light adaptation to 9500 millilamberts; closed circles, following light adaptation to 1700 millilamberts. Each point records the measurement with 8 retinas. The curve is drawn through the averages.



The data in Fig 4 agree with those of Riggs for the dark adaptation of *Rana pipiens*. The duration of the initial delay in visual purple accumulation is the same as the period elapsing before rod function begins. Moreover, the time required for complete regeneration is approximately the same as that found by Riggs for the attainment of a constant threshold in the frog.

These data also correspond to human dark adaptation, because the visual purple regeneration is delayed just as is the decrease in the rod threshold. However, the rod threshold reaches its minimum in about 25 to 30 minutes, while the visual purple maximum is reached at about 90 to 100 minutes. This difference in time is probably due to the difference in temperature between the

TABLE II

Changes in concentration of visual purple at 15° C in retinas of frogs after 10 minute light adaptation. Series I, light adaptation at 1700 millilamberts, Series II, 9500 millilamberts

Time in dark	Photometric density	
	Series I	Series II
<i>min</i>		
0	0.025	0.015
5	0.026	0.018
10	0.025	0.017
15	0.031	0.023
20	0.033	0.021
30	0.039	0.034
45	0.060	0.042
60	0.073	0.059
90	0.104	0.102
120	0.113	0.120

human and the frog measurements. The temperature coefficient of regeneration is about 1.8. Computed for 37.5° C, regeneration has approximately the same time characteristics as the human dark adaptation data.

Comparison with Zewi's measurements shows little agreement. Zewi reports no delay in visual purple regeneration following conditions of light adaptation similar to those here used. Thus his data correspond neither to frog dark adaptation (Riggs, 1936-37, and Granit, Munsterhjelm, and Zewi, 1939) nor to human dark adaptation. The occasional delays found by Zewi occur at low temperature, as is to be expected, but only following short periods of light adaptation. Computing these data to 37.5° C results in a negligible delay.

(c) *Light Adaptation*—The third series of measurements was made at one light intensity but with varying periods of light adaptation. They were under-

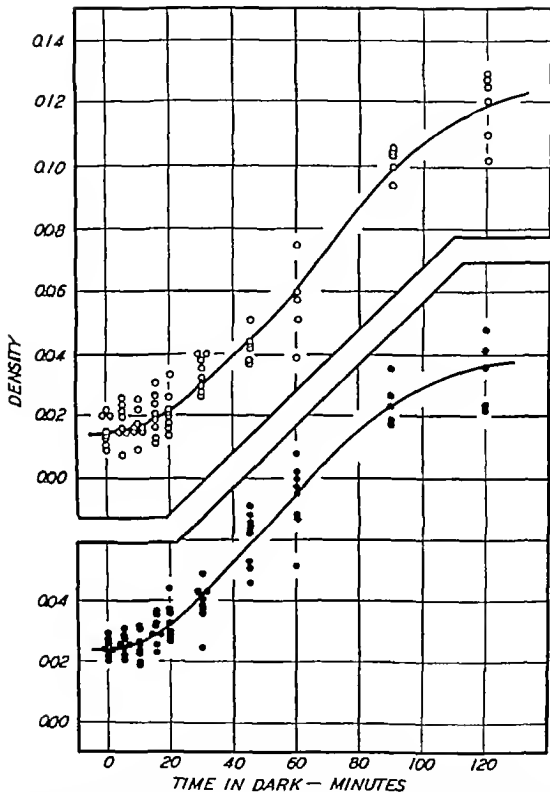


FIG. 4. Regeneration of visual purple in the intact eye of the frog at 15°C. The concentration is measured as density at 500 mμ. Open circles, visual purple concentration following light adaptation to 9500 millilamberts; closed circles, following light adaptation to 1700 millilamberts. Each point is from an extract of 8 retinas. The curves are drawn through the averages.

taken because of Zewi's failure to find delays in the regeneration of visual purple except following short periods of light adaptation

TABLE III

Changes in concentration of visual purple at 15° C in retinas of frogs after light adaptation to 9500 millilamberts Series I, 5 minute light adaptation, Series II, 20 minute light adaptation, Series III, 10 minute light adaptation

Time in dark <i>min</i>	Photometric density		
	Series I	Series II	Series III
0	0 029	0 011	0 015
5	0 033	0 011	0 018
10	0 038	0 013	0 017
15	0 041	0 025	0 023
20	0 043	0 029	0 021
30	0 057	0 051	0 034

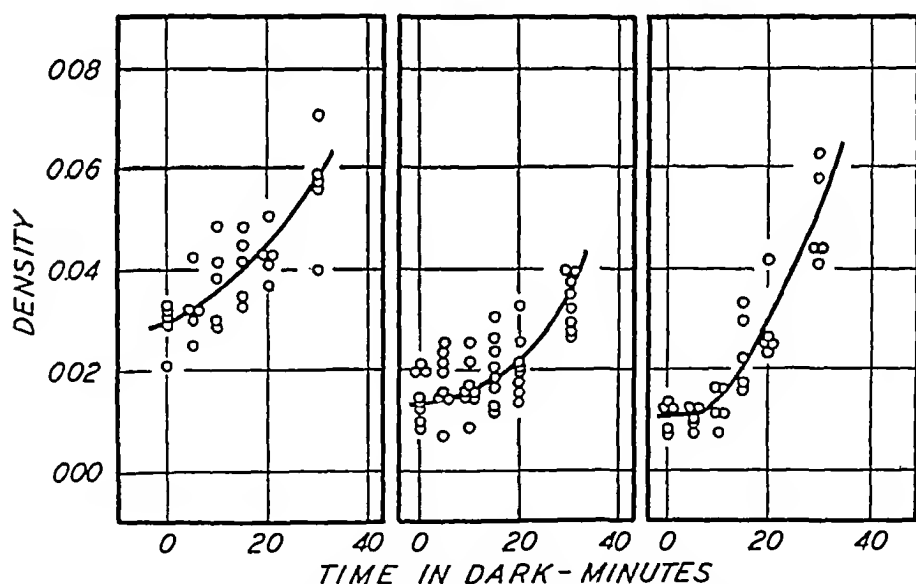


FIG 5 Regeneration of visual purple in the intact eye of the frog at 15°C following light adaptation to 9500 millilamberts Left, after 5 minutes of light adaptation, center, following 10 minutes light adaptation, right, following 20 minutes light adaptation

The procedure was as before Animals were light adapted at 9500 millilamberts, one group for 5 minutes and another for 20 minutes Dark adaptation occurred at 15° C, and was followed for the first 30 minutes only

The data are in Table III where each value represents the average of 5 measurements with 40 retinas The individual points are in Fig 5 Included

in the table and figure are the data from the previous experiment for light adaptation at 9500 millilamberts for 10 minutes at 15° C obtained under the same conditions of light and dark adaptation but at a different season.

The curves in Fig 5 show that the delay depends on the duration of light adaptation. Regeneration after 5 minute light adaptation begins immediately, whereas after 10 or 20 minute light adaptation, regeneration is delayed. Both delays are approximately the same, the 20 minute data showing a slightly longer one than the 10 minute data.

These experiments do not corroborate Zewi's measurements in which he finds that only short periods of light adaptation are followed by a delay in visual purple regeneration. The data agree with the work on human dark adaptation by Müller, by Johannsen, and by Wald and Clark, all of whom found that short light adaptation periods produce no delay in rod adaptation, while longer periods of light adaptation are followed by a delay before the increase in rod sensitivity begins. Only the data of Granit on frog retinal potentials show a delay in the recovery of sensitivity under all conditions of light adaptation.

## VI

### *Carotenoids*

The changes in the vitamin A and retinene concentrations were followed during dark adaptation. Estimation of the carotenoid content of the entire optic cup showed no regular changes in vitamin A or retinene. Consequently, only those retinas which could be entirely freed of pigment were used. The data are presented in Table IV and Fig 6. Each point represents the average of five measurements with 40 retinas.

The amount of vitamin A and retinene present in retinas dark adapted for 24 hours is also given in the table and indicated in the figure. We see that the amount of free retinene changes very little during dark adaptation at 25° C following 1700 millilambert light adaptation. The vitamin A content, however, is high at the conclusion of light adaptation and rapidly decreases until it shows little change after 60 to 90 minutes in the dark. The value obtained with completely dark adapted retinas indicates that vitamin A has not yet fallen to its final level during the period of the experiment.

Wald (1935, 1936) found that when isolated retinas are exposed to light, the visual purple bleaches and retinene appears. Upon continued illumination the retinene disappears and vitamin A appears in its place. This process takes about 1 hour. Our data show that in the eye of the intact animal a somewhat different series of events takes place. Even after 10 minutes of light adaptation, there is little retinene and much vitamin A in the retina.

The difference between isolated retina and intact animal does not lie merely in the possibility of the circulation removing the bleaching products of visual purple. If the failure of retinene to accumulate in the intact eye were due to

TABLE IV

Changes in concentration of carotenoids at 25° C in retinas of frogs after light adaptation to 1700 millilamberts for 10 minutes    Series I, retinene, 664 mμ, Series II, vitamin A, 612 mμ

Time in dark	Photometric density	
	Series I	Series II
<i>min</i>		
0	0 089	0 215
10	0 062	0 145
15	0 068	0 150
20	0 062	0 132
30	0 072	0 109
45	0 062	0 106
60	0 075	0 120
90	0 068	0 110
D A	0 059	0 086

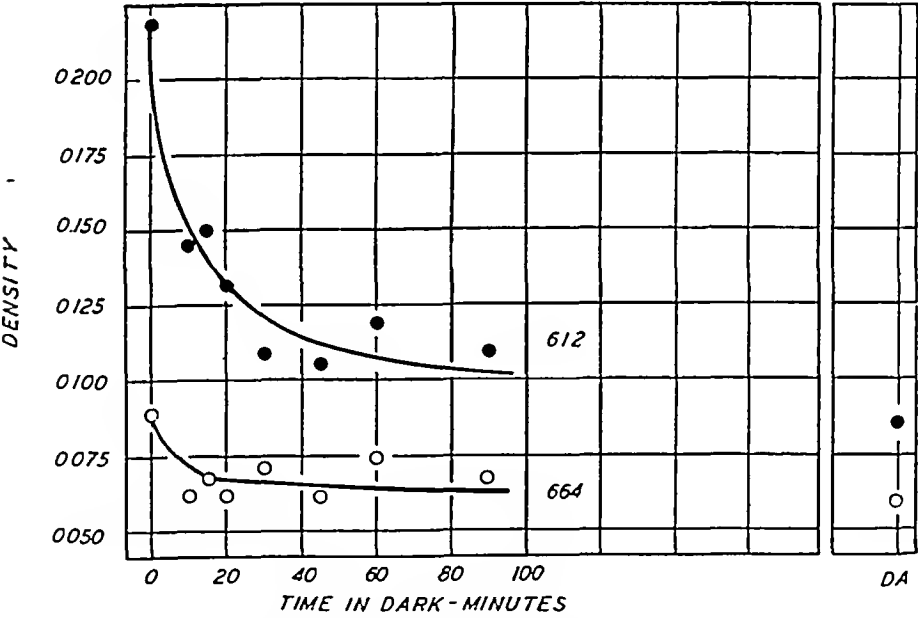


FIG 6 Changes in retinal carotenoids of the intact eye at 25°C following light adaptation to 1700 millilamberts    Open circles represent retinene, closed circles vitamin A

its removal by the blood system, it is to be expected that vitamin A would also be removed    It is therefore possible that the circulation in the eye permits a rapid conversion of retinene to vitamin A, or that vitamin A is formed directly from visual purple

## VII

*Kinetics of Visual Purple Regeneration*

The course of visual purple regeneration as shown in Figs 3 and 4 is sigmoid. A comparison of these data with those of dark adaptation can be made by plotting the dark adaptation data as the reciprocal of the threshold intensity against time, much as Piper (1903) did originally. This rests on the obvious

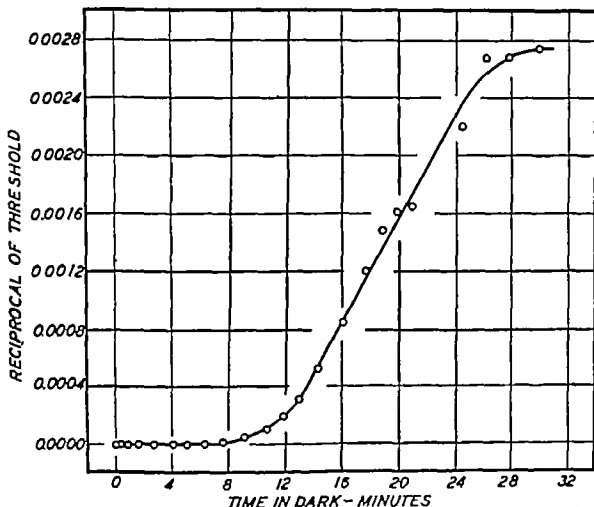


FIG. 7 Dark adaptation of human eye, data of Hecht, Haig and Chase (1937)

supposition that the concentration of sensitive material is inversely proportional to the intensity required to produce a constant photochemical effect. Fig 7 shows such a curve for human dark adaptation from the data of Hecht, Haig, and Chase, while Fig 8 shows a similar treatment of frog dark adaptation from the data of Riggs. Because of the ordinate scale, the separation of rod and cone function is not apparent. But neglecting the first few minutes of cone adaptation, one sees that the data show a slow rise in sensitivity (= concentration) followed by a rapid rise in a typical sigmoid manner. These curves are roughly similar to the regeneration curves of Figs. 3 and 4, and taken in conjunction with the similarity of effects produced in both cases by

temperature, and time and intensity of light adaptation, show that the process of dark adaptation depends upon the accumulation of visual purple in the retina

The curves in Figs 3 and 4 do not start from zero concentration, and the initial concentration following light adaptation to 9500 millilamberts is lower than that following adaptation to 1700 millilamberts. This difference between the two series, and to some extent the presence of an initial concentration following light adaptation is understandable in terms of the stationary state (Hecht, 1937)

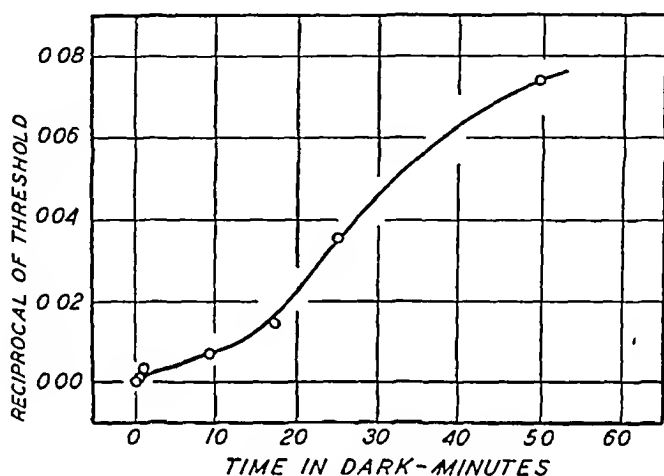


FIG 8 Dark adaptation of the frog eye measured by electrical method, data of Riggs (1936-1937)

When the photochemical system in the eye has reached a steady state, the concentrations are described by the equation

$$KI = x^n / (a - x)^m$$

where  $K$  is the equilibrium constant,  $I$  the light intensity,  $a - x$  the concentration of unbleached pigment,  $x$  the concentration of bleached products, and  $m$  and  $n$  the order of the reactions. It follows that the fraction  $x^n / (a - x)^m$  becomes larger as the intensity increases. Thus the higher the intensity, the smaller the amount of undecomposed photosensitive material remaining.

This expression also indicates that even at high intensities we can always expect to find a finite amount of photosensitive material in the retina. However, this amount is fairly small, and another factor is involved in producing residual visual purple in the light adapted retina. This is the fact that even with illumination from all sides, the entire surface of the retina is not exposed to light. This means that a small amount of visual purple, especially that in the region of the ora serrata, will not be bleached. Thus the apparent

initial concentration of visual purple is increased over that expected from stationary state considerations by a value which depends upon the area of the retina which is unbleached. Since the extent of the unbleached area remains sensibly constant by the use of identical procedures on all animals used, the concentration of visual purple is increased by a constant amount.

Attempts to fit theoretical equations to the data of visual purple regeneration have in the past been only partly successful. Tansley found that monomolecular and bimolecular equations described her data, while Zewi was unable to obtain a satisfactory theoretical description of his data. In both cases the data are too sparse for a critical test of the theoretical curves.

When visual purple is exposed to light, one or more photoproducts are produced. In the dark the decomposition products may recombine, probably with additional materials, to form visual purple again (*cf.* Hecht, 1937).

There are several possibilities which may be considered as the basis for a description of the kinetics of visual purple regeneration. The first is that it is a direct and simple transformation of one molecular species into another. The regeneration should then correspond to a simple monomolecular or bimolecular kinetics. The data in Figs. 3 and 4 exclude this possibility because of the sigmoid shape of the course of regeneration.

Two common chemical systems yield sigmoid curves for their kinetics. One is a catenary series of reactions in which an intermediate compound is formed between the beginning product and the end product. Such chemical reactions yield curves which are only slightly retarded at the beginning. Comparison of the relevant equations with the present data shows them to be inadequate in this respect. The regeneration of visual purple as recorded here has much too great an initial lag.

The other system producing a sigmoid kinetics is one involving autocatalysis. If we assume that the formation of visual purple is a direct chemical transformation which is catalyzed by visual purple itself, we can describe the present data adequately by the usual equation for such an autocatalyzed system. The general equation describing the process is

$$dx/dt = k_1 x^m (a - x)^n$$

where  $k_1$  is the velocity constant,  $(a - x)$  the concentration of visual purple at moment  $t$ ,  $a$  the final concentration of visual purple,  $x$  the concentration of photoproduct, also at moment  $t$  and  $m$  and  $n$  the order in which  $(a - x)$  and  $x$  enter the reaction. Taking  $m$  and  $n$  as 1, and integrating, the equation yields

$$k_1(t - t_{1/2}) = \frac{1}{a} \log \frac{a - x}{x}$$

where  $t_{1/2}$  is the time when the reaction is half complete. Other values of  $m$  and  $n$  may be tried, but for the present the value of 1 is sufficient.



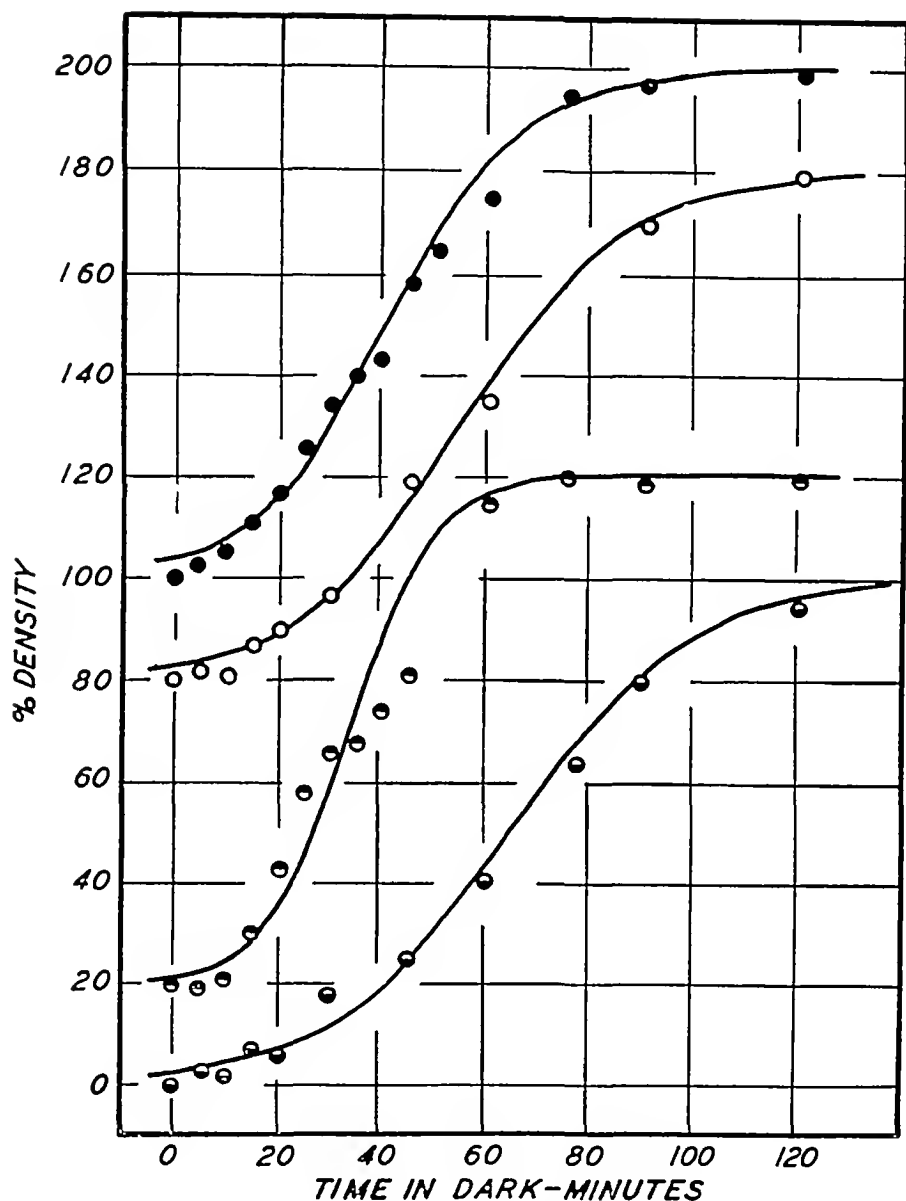


FIG 9 Computation of visual purple regeneration in terms of the equation for an autocatalyzed chemical reaction. The data are those of Figs 3 and 4. The three upper curves and data are displaced 20, 80, and 100 per cent units upward.

Fig 9 shows the application of the integrated equation to the measurements. The data are plotted on the basis of a change in visual purple concentration from 0 to 100 per cent. Since the concentration of visual purple is never zero after the completion of light adaptation, this residual visual purple con-

centration has to be eliminated as a factor in computing the data. The density at zero time of dark adaptation is therefore subtracted from every other density determination of the series. The per cent of the maximum density is then computed for each point, thus giving a series which varies from 0 to 100 per cent.

In Fig. 9 the curve of the autocatalyzed monomolecular equation is drawn with specific values of the velocity constant  $k_1$  for each series of data. It is apparent that the measurements are adequately described by the equation. The slopes are different for the different series. In particular, it is to be noted that  $k_1$  is greater at 25°C. than at 15°C. in accordance with general knowledge.

The precise chemical meaning of the autocatalyzed reaction may really be that visual purple, or perhaps another material formed in equivalent amount as visual purple regenerates, catalyzes the formation of visual purple from the material present in the light adapted retina. At present it is too soon to say. That the equation is first order merely may mean that the other precursors are present in excess, or it may mean that no great chemical change is involved. This is in keeping with the work of Hecht and Pickels (1938) which showed that the bleaching of visual purple in solution corresponds to only a slight change, if any, in the size of the molecule.

#### SUMMARY

1 The accumulation of visual purple in the retina after bleaching by light has been studied in the intact eye of the frog. The data show that duration and intensity of light adaptation, which influence the course of human dark adaptation as measured in terms of visual threshold, have a similar influence on the course of visual purple regeneration.

2 At 25°C. frogs which have been light adapted to 1700 millilamberts and then placed in the dark, show an increase in visual purple concentration which begins immediately and continues for 70 minutes until a maximum concentration is attained. The increase, although beginning at once, is slow at first, then proceeds rapidly, and finally slows up towards the end. Frogs which have been adapted to 9500 millilamberts show essentially the same phenomenon except that the initial slow period is strongly delayed so that almost no visual purple is formed in the first 10 minutes.

3 At 15°C. the initial delay in visual purple regeneration occurs following light adaptation to both 1700 and 9500 millilamberts. The delay is about 10 minutes and is slightly longer following the higher light adaptation.

4. The entire course of visual purple accumulation in the dark takes longer at the lower temperature than at the higher. The temperature coefficient for 10°C. is about 1.8.

5 In contrast to the behavior of the isolated retina which has small amounts

of vitamin A and large amounts of retinene immediately after exposure to light, the intact eye has large amounts of vitamin A and little retinene after exposure to light for 10 minutes. In the intact eye during dark adaptation, the amount of vitamin A decreases markedly while retinene decreases only slightly in amount. If retinene is formed in the intact eye, the change from retinene to vitamin A must therefore occur rapidly in contrast to the slow change in the isolated retina.

6 The course of visual purple regeneration may be described by the equation for a first order autocatalyzed reaction. This supposes that the regeneration of visual purple is catalyzed by visual purple itself and accounts for the sigmoid shape of the data.

It is with deep appreciation that the author wishes to acknowledge the willing and generous guidance of Professor Selig Hecht.

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## HUMORAL RHEOLOGY

### I VISCOSITY STUDIES AND ANOMALOUS FLOW PROPERTIES OF HUMAN BLOOD SYSTEMS WITH HEPARIN AND OTHER ANTICOAGULANTS

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The effect of anticoagulants on the flow properties of blood, plasma, and serum has been subjected to investigation with various results. Trevan (1) studied the viscosity of blood with various anticoagulants. He found that hirudin has no appreciable influence on the viscosity, whereas oxalate, citrate, and fluoride in the solid state were inadmissible because they altered the volume of the corpuscles by osmotic changes. Oberst (2) has recently stated that heparin produces an increase in the consistency of plasma and serum whereas potassium oxalate produces no change. Eckstein, Book, and Gregg (3) reported that heparin, *in vitro* or *in vivo*, has not been observed to alter the blood viscosity significantly. Our purpose was to discover whether various units of purified heparin, added to blood and serum, would alter their viscosities.

In the course of these studies it became apparent that anomalous flow properties of the blood contributed to the complexity of the problem. These anomalies have been referred to in the literature. Dow and Hamilton (4) occluded the aorta of dogs and studied the curve of declining pressure, measured peripherally to the point of occlusion. At a pressure of about 20 mm. of mercury the flow of blood stopped. They attributed this phenomenon to the anomalies of the apparent viscosity of blood, thus agreeing with the findings of Whittaker and Winton (5) who studied the apparent viscosity of blood suspensions. The latter found this phenomenon in glass viscometers as well as in the biological viscometer which they developed in the isolated hind limb of the dog. This same phenomenon was also found following arterial occlusion by employing a piezographic sphygmomanometer by Lahy (6) who estimated that a minimum pressure between 5.7 and 8.2 mm. of mercury will maintain circulation.

The similarity of these phenomena to those studied and analyzed in other fields of rheology, where relationships have been developed, justified an attempt to apply these relationships to the blood anticoagulant systems.

#### *Rheological Nomenclature*

As the systems studied do not conform to the classical concept of fluid flow, some of the accepted concepts for such anomalous systems have been

adopted to the extent permitted by the limitations in the work done and the general knowledge of the subject

The flow properties of blood systems do not conform to the Newtonian hypothesis that the rate of shear and the applied stress are proportional over a wide range as is indicated by the following equation

$$\eta = \frac{S}{R} \quad (1)$$

in which

$R$  = the rate of shear,

$S$  = the shearing stress,

$\eta$  = the proportionality factor (viscosity)

Only systems whose flow properties obey this proportionality as shown in Equation 1, and graphically in Fig 1, Line I, are true fluids and are said to exhibit true viscosity. Only to such systems can the term "viscosity" be strictly applied.

For the more complex systems, not conforming to Equation 1, several relationships have been developed. The simplest of these is the Bingham concept (7) graphically shown by the flow diagram in Fig 1, Line II, and by the following equation

$$\eta_R = \frac{S - S_0}{R} \quad (2)$$

in which

$R$  = rate of shear,

$S$  = shearing stress,

$S_0$  = Bingham "yield value,"

$\eta_R$  = proportionality factor with the same dimensions as viscosity, but defined as pseudoviscosity as it does not fill the full requirements of Equation 1

The data presented by Whittaker and Winton conform to the Bingham type of flow diagrams. That their plot of pressure *versus* rate of flow was linear with an intercept on the pressure ordinate indicated that there, apparently, was a definite minimum pressure below which the blood would not flow. The simple Bingham flow diagram and concept were accepted as most suitable for our study, in view of the preceding evidence and because the deviation for the data reported here from the Bingham relationship is not large. Further, the quantity of data available does not justify the application of more complex, possibly more exact formulae.

The simple term "viscosity" in the subsequent report will be applied only to systems which conform to Equation 1, and whose viscosities do not change

with changes in the applied stress. Its flow diagram is characterized by a straight line through the origin, as shown in Fig 1, Line I. Its magnitude is proportional to the slope of the angle "V"

The term "apparent viscosity" will be applied to values obtained in terms of viscosity for non Newtonian systems at a given stress. These values are obtained relative to water, or standard glycerol water mixtures, of known viscosity, tested under the same conditions, using as a factor their viscosities in centipoises. In this respect, "apparent viscosity" here used differs from "apparent viscosity" as defined by Winton and Bayliss (8) and Whittaker and

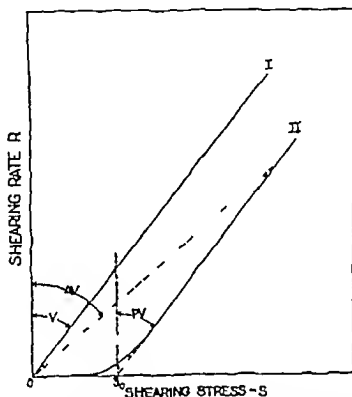


FIG 1 Flow diagram for true liquid flow (Line I) and anomalous flow (Line II) Angle V is proportional to viscosity AV and PV are proportional to apparent viscosity and pseudoviscosity respectively intercept  $S_0$  is defined as yield value

Winton, as we believe their water factor was unity rather than the viscosity of water in centipoises. It is only necessary to multiply our 'apparent viscosity' by 0.68 (the viscosity in centipoises of water at 37.5°C) to obtain the Whittaker Winton Bayliss "apparent viscosity" which is the equivalent of our "apparent specific viscosity". The term "specific" is introduced because it is common usage to apply it to the values relative to water.

The "apparent viscosity" is proportional to the tangent of the angle "AV" of Fig 1. Its value is dependent on the stress on Line II to which the dotted line is drawn. If the determination is made at a low stress the angle AV is large and the apparent viscosity is higher. If the determination is made under



a high stress the angle  $AV$  is smaller, and the viscosity, consequently, is smaller. This is in keeping with the observations of Whittaker and Winton.

"Pseudoviscosity" will be the expression applied to the Bingham type of non-Newtonian fluid, and is graphically proportional to the angle  $PV$  of Fig. 1. Its magnitude, in terms of centipoises, is determined by the use of Fig. 2. This value is the reciprocal of the Bingham "mobility." A pseudoviscosity determination requires a flow diagram of rate *versus* at least two, preferably three or more, stresses. In each instance these diagrams were drawn to a uniform scale. This uniform scale was used in the development of Fig. 2.

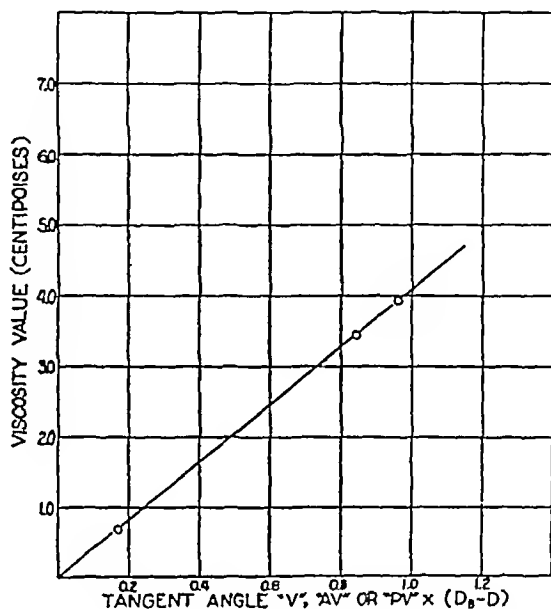


FIG. 2 Calibration chart, viscosity value in centipoises *versus* tangent of flow diagram line angle (corrected for density)

The expression "yield value" is used to describe the intercept on the stress ordinate, in this instance, the sine of the angle of viscometer tube inclination. The values given are based on the fact that the stress causing the ball to roll is directly proportional to the sine of the angle of the viscometer tube ( $\beta$ ) and the difference in the density of the ball and fluid ( $D_B - D$ ) multiplied by the gravitational force 980 (to convert it to dynes) as follows:

$$\text{Yield value (dynes)} = 980 \sin \beta (D_B - D) \quad (3)$$

The yield values reported were not expressed in the conventional dynes per square centimeter as the available data do not permit an expression of the stress intensity. The value reported is proportional to the yield value, or shearing strength (9). For similar reasons, the yield values reported by Whittaker and Winton and Dow and Hamilton are functions of the stress intensity.

Accordingly, the various values reported, although proportional in some fashion, are not subject to a direct comparison.

For the Bingham type of flow, the system is completely defined by the factors pseudoviscosity and yield value. This is the minimum number of constants capable of completely defining the flow properties of the anomalous systems observed, and is necessary for the solution of Equation II.

#### EXPERIMENTAL

*1 Apparatus*—A falling ball viscometer, modified to allow the determination of flow properties at several stresses was accepted as most suitable for the problems involved. This choice was made for the following reasons: (a) the viscosity determination of a given system at various rates of shear can be made with ease and rapidity without reassembling the instrument, (b) ease of subjecting a given specimen to various and repeated agitations which are necessary for the study of thixotropy and dilatancy, (c) ease of cleaning the instrument, and (d) ease of obtaining and maintaining constant temperatures. In addition, because various investigators attribute the anomalies observed to the viscometer it was desirable to establish whether these anomalies still exist with an entirely different type of instrument.

The primary limitations of this apparatus are: (a) approximately 6 cc. of specimen are required for any given series of tests, (b) the flow conditions do not simulate those existing in the arterial and venous systems, (c) the yield values reported are only proportional to the stress intensity required to cause flow, (d) the rates of ball travel must not exceed 0.30 cm. per second (equivalent to approximately 0.5 cc. per second) to avoid turbulence and consequently only a five-fold change in applied stress is convenient. These important limitations of the instrument will require eventual consideration, but were not believed significant in this exploratory study.

The flow diagram of rate versus shearing stress expressed as the sine of the angle of tube inclination, for a true liquid, (Fig. 3) is a straight line over the range used and passes through the origin. This indicates that no objectionable variables are introduced. Similarly the slopes from this flow diagram for water and two known glycerol-water solutions corrected for density, plotted against their corresponding viscosities, are straight lines through the origin (Fig. 3).

Fig. 4 shows the 'rolling ball viscometer' assembled. It consists of a precision 8.3 mm. bore glass tube (section of a 10 cc. precision pyrex pipette) filled with the liquid in question. In this viscometer tube rolls a precision, highly polished pyrex sphere of 7.94 mm. diameter. This assembly is fastened to predetermined reproducible angles made available by the various pegs shown on the stand. The stand with the tube fastened at the desired angle with rubber bands is set on a level plate glass platform, and submerged in a transparent water bath with adequate lighting from below.

The rate of the ball's roll is dependent on the tube angle, the viscosity of the liquid, the differences in the densities of the ball and liquid and the respective radii of the tube and the ball. Accordingly by holding all the variables other than the liquid constant, and recording the rate of ball travel relative to that of a sample of known viscosity the viscosity of an unknown can be determined on a relative basis. The

standards were distilled water and water-glycerol solutions of known viscosity (10) and known density (11)

When flow diagrams were not prepared as a matter of convenience, or when flow properties were determined at a single shearing stress, the viscosities or apparent

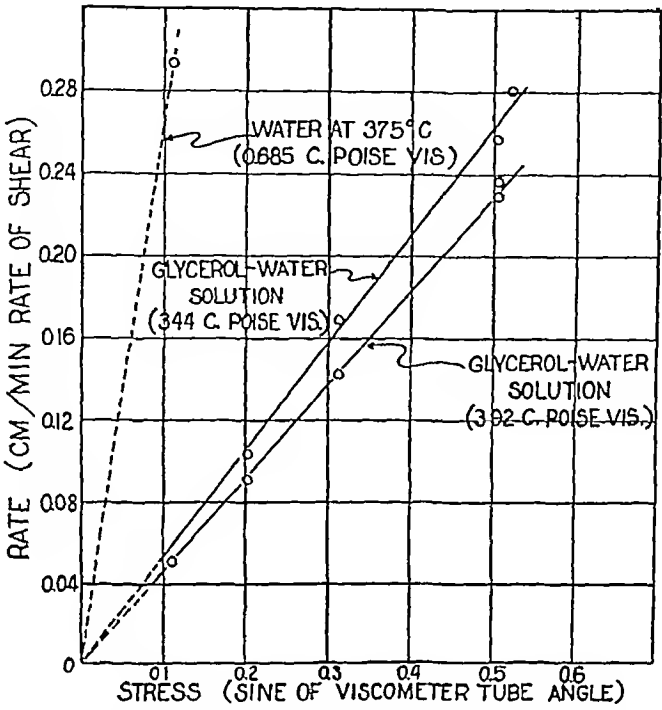


FIG 3 Flow diagram obtained with rolling ball viscometer for standard viscosity liquids exhibiting true viscosity

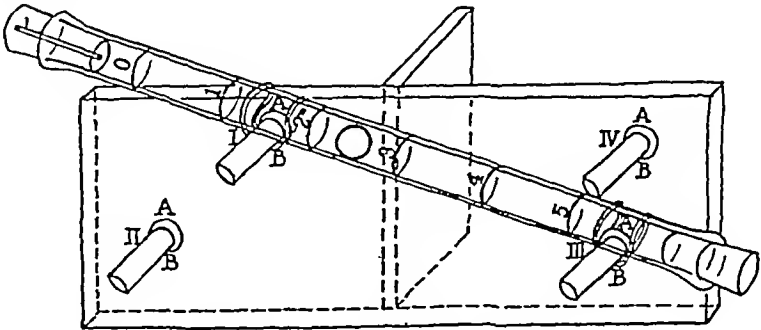


FIG 4 Rolling ball viscometer

viscosities were calculated A modification of the conventional specific viscosity formula was used, employing an expression which corrected for differences in the densities of the ball and the liquid The following equation was used

$$\frac{\eta_1}{\eta_2} = \frac{T_1(D_B - D_l)}{T_2(D_B - D_l)} \tag{4}$$

in which

- $\eta_1$  = viscosity in centipoises of the standard liquid,
- $\eta_2$  = viscosity of the unknown system,
- $T_1$  = time in seconds necessary for the sphere to travel the predetermined distance in the standard liquid at a given tube angle,
- $T_2$  = time in seconds for the sphere to travel through the unknown under conditions identical to  $T_1$ ,
- $D_s$  = density of the ball (2.24),
- $D_1$  = density of standard liquid,
- $D_2$  = density of unknown system.

For the blood-anticoagulant studies with several shearing stresses which permitted the development of a flow diagram the data were (a) plotted on uniform ordinates (b) the slope of the flow line determined, (c) the slope corrected for density by multiplying the factor  $(D_s - D_2)$ , and (d) the viscosity value obtained by use of the calibration curve (Fig. 2)

When flow diagrams were constructed for systems exhibiting anomalous flow properties, at least two, preferably more, shearing stresses were required. This was possible with a given filling of the instrument by merely changing the angles of the tube. The angles used were  $11^\circ 41'$ ,  $18^\circ 11'$ ,  $30^\circ 25'$ , and  $31^\circ 33'$ . The application of the inclined plane principle indicates that the resulting shearing stresses are directly proportional to the sine of the angle. Accordingly, a plot of the sine of the angle of tube inclination *versus* the rate of the ball travel results in the desired flow diagram of rate *versus* stress. That this is valid is indicated by the linear plot of viscosity *versus* slope, passing through the origin obtained on two standard liquids known to exhibit true viscosity as in Fig. 3.

The reproducibility for a given filling and position with either a true liquid or non-Newtonian system is well within 1 per cent. Duplicate determinations for subsequent fillings agree well within 3 per cent for true liquids, and this agreement for blood anticoagulant systems is about 5 per cent.

The operation of the viscometer consists of inclining the tube and stand to force the ball to roll to the upper end of the tube. The stand is then set on the level platform and the ball rolls down the desired incline. The time necessary for the ball to pass two predetermined points is then determined. The distance between the points was 7.408 cm. the equivalent of 4 cc. as indicated by the original pipette graduations. Determinations near either extremity of the tube were avoided to minimize the possibility of end-effects.

Precautions were necessary to exclude any air bubbles from the system. The stopper with the capillary permitted the escape of such bubbles while closing the system. It was also necessary to avoid the formation of air bubbles in the system after closing, due to the air and other gases coming out of solution. Boiling the distilled water used in the standard eliminated this. It was found essential that the ball and tube be rigorously cleaned. The ball, once clean and dry, was always handled with rubber tipped forceps.

The second stopper shown in the diagram was included to facilitate the tube cleaning operation. Care was taken not to scratch the tube bore or the ball.

*2 Preparation of Systems*—Blood was obtained from the antecubital veins

of three humans and diluted 9:1 with the anticoagulants dissolved in distilled water. The viscosities of the blood systems were determined for 2 u, 5 u, 25 u, and 125 u of heparin (Connaught Laboratories, University of Toronto, and Lederle Laboratories Pearl River, New York, 110 Murray-Best units per mg. of the sodium salt) per cc., and 0.2 per cent sodium oxalate, 0.2 per cent potassium oxalate, and 0.3 per cent sodium citrate per cc. A portion of each blood-anticoagulant sample was used for the determination of the densities, using a Nicol tube pycnometer. After these determinations, these systems were centrifuged at 3000 R.P.M. for 20 minutes. The supernatant plasma was decanted and viscosities and densities determined. Following withdrawal of the blood, all tests were run continuously within 6 hours. Samples containing low unitage were tested first.

### OBSERVATIONS

#### *A The Influence of Various Units of Heparin upon the Plasma and Blood Apparent Viscosities*

Columns 1 and 5 and columns 2 and 6 of Table I cover the data on the apparent viscosities of the blood and plasma of two humans at relatively low rates of shear. As can be seen, the apparent viscosities of blood and plasma are always decreased with higher amounts of heparin, although not in direct proportion. 2 u, 5 u, and 25 u per cc. heparinized human C blood and plasma always exhibit higher apparent viscosities than the corresponding 0.2 per cent sodium oxalate and 0.3 per cent sodium citrate systems. Only the 2 u heparin-blood and heparin-plasma systems for human A have a higher apparent viscosity than the oxalate-blood and oxalate-plasma systems. It may be noted that similar effects were observed with heparin on the viscosities of serum-anticoagulant systems. Sera were obtained from 2 humans, 2 dogs, and 1 sheep and diluted 14:1 and 13:2 with various units of heparin dissolved in distilled water. The lowering effect of heparin on the viscosity was always present, although not in direct proportion to the amount of heparin added.

#### *B The Relationship between Blood and Plasma Viscosities*

The  $\frac{\eta}{\eta_0}$  ratio, in which  $\eta$  = viscosity of blood and  $\eta_0$  = viscosity of plasma, was found constant within the limits of the instrument and the type of systems studied (Table I). Some peculiarities do exist, in human C there was ratio increase with higher heparin concentrations, whereas in human A, the reverse trend was noted. In the fourth and eighth columns of this table the volume of the corpuscles is shown as calculated from the formula of Whittaker and Winton

$$\Phi = \left(1 - \frac{k\eta_0}{\eta}\right)^3 \quad (5)$$

in which  $100\Phi$  = volume of corpuscles as per cent of total volume of blood, derived from the formula of Hatschek (12),  $\eta = \frac{\eta_0}{1 - \Phi^{1/3}}$

The factor  $k = 0.6$  was introduced by Whittaker and Winton for corpuscular concentrations within the limits of 10 to 80 per cent, since Hatschek's formula only holds for suspensions above 60 per cent and, after Trevan, in blood suspensions above 45 per cent. Whittaker and Winton claim that the constant,  $k$ , is increasing with pressure if the pressure is low, but approximately equal to 0.6 over a wide range of higher pressures such as obtained in high velocity type of viscometers. Our viscometer, although not of a capillary type, involves a high velocity, and we therefore felt justified in using the empirically found factor of Whittaker and Winton. As can be seen from the table, for human C the corpuscular volume varies between 54.5 per cent and 61.0 per cent and for human A, between 44.0 per cent and 48.7 per cent. Human C shows

TABLE I

*The Relationship between Blood and Plasma Apparent Viscosities (Viscosities Determined at 37.5° C. and at an Angle of 31°33')*

Per cc. blood-anticoagulant mixture 9:1	Human A				Human C			
	Blood $\eta$	Plasma $\eta$	$\frac{\eta}{\eta_0}$	100%	Blood $\eta$	Plasma $\eta$	$\frac{\eta}{\eta_0}$	100%
	centipoises	centipoises		per cent	centipoises	centipoises		per cent
Heparin 2 u	5.61	1.99	2.82	48.7	4.87	1.49	3.27	54.5
Heparin 5 u	5.27	1.93	2.73	48.1	4.73	1.43	3.31	55.0
Heparin 25 u	5.03	1.95	2.58	45.1	4.61	1.36	3.39	55.7
Heparin 125 u	4.87	1.93	2.53	44.0	4.31	1.25	3.45	56.3
Sodium oxalate 0.2 per cent	5.49	1.96	2.80	48.6	4.32	1.25	3.46	56.6
Potassium oxalate 0.2 per cent	5.46	1.96	2.79	48.2				
Sodium citrate 0.3 per cent					4.46	1.13	3.95	61.0

100% = calculated corpuscular concentration.

increases in the corpuscular concentration whereas human A shows decreases with increasing amounts of heparin. We have not compared these values with hematocrit values obtained by centrifugation of the blood, although it may be assumed that the amount of corpuscles in systems from the same blood with drawal should be the same, since the proportions of the dilutions are identical.

### C The Anomalous Flow Properties of Blood Anticoagulant Systems

Table II summarizes the values obtained for three human bloods for the apparent viscosity and apparent specific viscosity. These values progressively decrease with increases in the stress as is to be expected of such non-Newtonian fluids. Table III summarizes the pseudoviscosity values and yield values for these systems. The flow diagram obtained for three or more different stresses for the 0.3 per cent sodium citrate per cc. and the 25 u of heparin per cc. of human A is shown in Fig. 5. It is apparent that the data fall closely to a straight line, which on extrapolation intercepts the stress ordinate, demon-

TABLE II  
*Apparent Viscosity and Apparent Specific Viscosity of Blood Systems*

Anticoagulant per cc.	Tube angle	Apparent viscosity (expressed in centipoises)			Apparent specific viscosity (relative to water as unity)		
		Human A	Human B	Human C	Human A	Human B	Human C
Heparin 2 u	18°11'	5 70	4 97		3 88	3 38	
	31°33'	4 68	4 74		3 18	3 22	
Heparin 5 u	11°41'			5 42			3 69
	18°11'	5 28	4 81		3 59	3 28	
	30°25'			4 28			2 91
	31°33'	4 46	4 40	4 12	3 03	2 99	2 80
				4 12			2 80
Heparin 25 u	11°41'	6 02		4 80	4 08		3 27
	18°11'	5 10	4 69		3 47	3 19	
		4 90		4 58	3 33		3 12
	30°25'			3 87	3 25		2 63
	31°33'	4 30	4 32	3 86	2 92	2 94	2 62
		4 07		3 74	2 77		2 54
Heparin 125 u	18°11'	4 95	4 36		3 37	2 97	
	31°33'	4 07	4 15		2 77	2 82	
Potassium oxalate 0.2 per cent	18°11'	5 55	5 40		3 77	3 67	
	31°33'	4 62	4 70		3 14	3 20	
Sodium oxalate 0.2 per cent	18°11'	5 02	4 38		3 42	2 98	
	31°33'	4 27	4 05		2 90	2 76	
Sodium citrate 0.3 per cent	11°41'	5 57			3 79		
	18°11'	4 46			3 04		
	30°25'	3 91			2 66		
	31°33'	3 76			2 56		

For a given condition, the second value corresponds to datum from a second blood withdrawal after 4 days

TABLE III  
*Pseudoviscosity Values and Yield Values of Human Blood Systems*

Anticoagulant per cc.	Pseudoviscosity (expressed in centipoises)			Yield value in dynes		
	Human A	Human B	Human C	Human A	Human B	Human C
Heparin 2 u	3 67	4 12		126	67	
Heparin 5 u	3 55	3 90	3 66	121	70	76
Heparin 25 u	3 46	3 85	3 38	118	67	71
	3 32*			109*		
Heparin 125 u	3 29	3 80		115	49	
Potassium oxalate 0.2 per cent	3 65	4 03		122	81	
Sodium oxalate 0.2 per cent	3 50	3 60		103	63	
Sodium citrate 0.3 per cent	3 30			93		

\* = Value from second withdrawal after 4 days

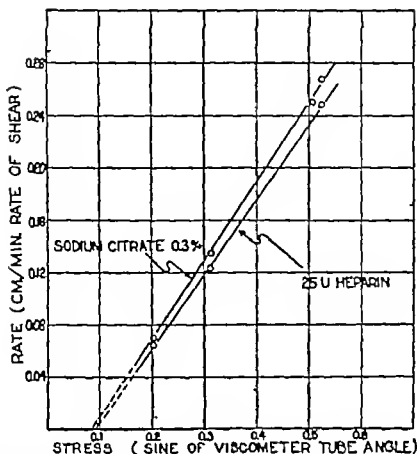


FIG 5 Flow diagram for human A blood with sodium citrate and heparin as anticoagulants exhibiting Bingham type of anomalous flow

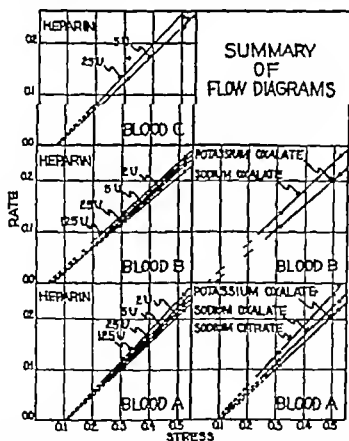


FIG 6 Flow diagram of humans A, B, and C bloods with various anticoagulants. Shearing stress as sine of angle of tube inclination versus rate of shear as centimeters per minute of sphere travel.



strating that the Bingham treatment of anomalous flow approximately fits these systems. The flow diagrams of Fig 5 are the most consistent obtained. The remaining data summarized in Fig 6, although not as precise, in each instance confirm the general observations made regarding Fig 5. These data primarily consist of a straight line, drawn exactly through two points corresponding to values obtained for two stresses. A progressive shift occurs with trends toward lower yield values and lower pseudoviscosity with increased heparin concentration.

#### *D Thixotropy and Dilatancy*

Exploratory results in this complex field are summarized by Fig 7. The system studied, containing 5 u of heparin per cc had been allowed to stand 6

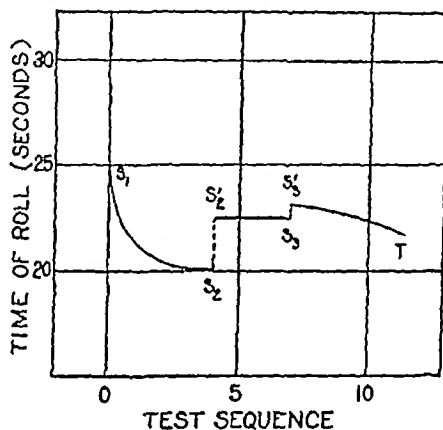


FIG 7 Change in consistency due to agitation and standing as measured by the time required for the ball to pass two fixed points

hours, at which time a small clot was removed, and the viscometer filled and tests started.

The progressive decrease in the time of roll  $S_1$  to  $S_2$  with the continued gentle agitation resulting from the passage of the ball, indicates a thixotropy-like phenomenon. On severe agitation the time of roll increased as indicated by  $S_2$  to  $S_2'$ . This increase has the general appearance of dilatancy. The gradual decrease from  $S_2'$  to  $T$ , 23.2, 23.0, 22.8, 22.8, and 21.6 could be attributed to thixotropy that is not entirely reversible.

The gradual average increase in the rolling time noted when comparing value  $S_2$  to  $T$ , is believed to be age-hardening, a phenomenon demonstrated by an increase in the viscosity values found for several of the systems that were allowed to stand after the initial determinations were completed.

#### DISCUSSION

The data obtained agree with those of Whittaker and Winton (5) and Dow and Hamilton (4) in that the blood systems studied exhibited anomalous flow

properties. Figs 5 and 6 offer much the same type of flow diagram as that of the Whittaker Winton biological viscometer, being a straight line, which, on extrapolation, intercepts the stress ordinate. Similarly, they are in general agreement with the data of Dow and Hamilton and of Lahy (6) who found that a distinct pressure is necessary to cause flow. On the basis of these three different experiments, and the data here offered, it appears certain that the yield value phenomenon exists in the blood systems studied and that to completely characterize the flow properties of such systems, the yield value must be taken into consideration.

We believe the data presented indicate that the Bingham treatment (Equation 2) can be used for this purpose, and that its conditions are reasonably well filled by the data of Winton and Whittaker as well as by the data presented in Figs 5 and 6 of this article. It is quite possible that further study will develop information indicating that the flow of these blood systems, particularly at a very low stress, is not linear and that the yield value obtained by the simple Bingham concept may be too high. For such systems a more exact relationship might be that of Herschel and Bulkley (13) or some similar variation (14), including an exponential factor. However, it is believed the limitations of the available data did not warrant the present application of these more complex treatments.

From the experiments summarized in Tables I and II it may be concluded that heparin tends to decrease the viscosity or apparent viscosity of blood and plasma. No mechanism can be offered at this time capable of explaining this decrease. The decrease in density found in the course of this investigation (15) contributes to the complexity of the phenomenon. Further investigation is required before a satisfactory explanation can be offered.

Table III indicates that the constants for the Bingham flow type of system, namely, pseudoviscosity and yield value, gradually decrease with increasing heparin concentrations.

It is conceivable that with some systems, one of these values would be materially changed while the other remains essentially the same. An indication of this results from a comparison of the flow properties of human A blood from the same withdrawal with 125 u heparin per cc. and 0.3 per cent of sodium citrate per cc. Although the pseudoviscosities are identical, the yield values differ by 20 per cent. This lower yield value accounts for the lower apparent viscosities exhibited by the sodium citrate-blood systems. By the same process a comparison of 5 u heparin added to human A and human C bloods per cc. indicates that the apparent viscosity of human A is approximately 8 per cent higher than human C, while the pseudoviscosity is some 3 per cent lower. This discrepancy is only understandable after the yield value is taken into consideration and it is realized that its value for blood system A is 40 per cent higher.

A study of Table III leads to the belief that the yield value tends to be

more specific for particular bloods than either the apparent viscosity or the pseudoviscosity. For example, the yield values of the various anticoagulant-blood systems of human A, irrespective of type of anticoagulant or concentration of heparin agree among themselves, on the average,  $\pm 9$  dynes. Blood B systems under similar conditions agree to  $\pm 7$  dynes. Accordingly, the order of agreement of the two systems is approximately the same. However, comparing them to each other they differ by approximately 51 dynes, or on a percentage basis, human B has 34 per cent to 58 per cent lower yield value. A similar comparison of the pseudoviscosities indicates that human A blood systems have, on the average, a 9 per cent lower value. Because of these observations, it is believed that the Bingham treatment may characterize the flow properties of blood and that the two constants, pseudoviscosity and yield value, for any one of these systems, may prove to be of physiological significance. This seems particularly true of the yield value.

In Table I, columns 4 and 8, for human A and human B blood systems verify the findings of Trevan (1) and Whittaker and Winton in that the ratio of the apparent viscosity of the blood and plasma systems is reasonably constant. Whether this ratio can be applied successfully to the calculations of the corpuscular concentration is questionable. As the apparent viscosity varies radically with the instrument and the rate of shear, the ratio of the blood and plasma viscosities will vary, and accordingly, whatever modification of the Hatschek formula is adopted will be dependent specifically on the test conditions under which it was developed. Subsequent use would require determinations under identical conditions. A possible exception, as noted by Whittaker and Winton, would be determination of the viscosity at very high shearing stresses where the yield value would be a minor variable in the apparent viscosity value.

Whittaker and Winton report that glass viscometers, even of the high velocity type give entirely too high a value for the apparent specific viscosity ("apparent viscosity" using the Whittaker-Winton-Bayliss terminology). In Table II, columns 4, 5, and 6 offer apparent specific viscosity data obtained by the modified falling ball instrument. At higher shearing stresses, values of  $2.5+$  are obtained which approach the  $2.2 \pm 0.2$  reported by Whittaker and Winton on the basis of their dog hind limb viscometer.

Since one of us (16) found that heparinized blood and plasma exhibit the phenomenon of thixotropy, the rolling ball viscometer was used to demonstrate it. The thixotropy and dilatancy data summarized in Fig. 7 exhibit the complexity of this phase of the problem. It appears that, although thixotropy is present, it is not entirely reversible, as required by the classical definition. This seems to be due in part to two other phenomena: (a) dilatancy, (b) age-hardening.

In 1936 and 1938 Freundlich *et al.* reported the phenomenon of dilatancy in systems of microscopical as well as of ultramicroscopical particles in suspension.

(17, 18) The particles behave as normal fluids at a low rate of deformation, because there they have sufficient opportunity to slip over each other. However, by fast deformation the resistance against deformation is rapidly augmented and a phenomenon occurs which compensates the deforming stress. The particles are accumulated locally and have no longer the ability to pass each other. Thus these systems behave as solids. As soon as the external forces cease acting, with spontaneous restoration of the regular distribution of the particles, they again form a liquid system. This rheological phenomenon is also referred to as "snow plough effect" by Jordan (19) who studied it in smooth muscles. To our knowledge this phenomenon was not described with blood systems. Like the phenomenon of thixotropy it can be measured with our viscometer on a relative basis.

The age hardening phenomenon, that is, the increase of viscosity of blood systems on standing although not discussed in detail in the paper, has been definitely established on a few specimens. Its extent and rate have not been established. The occurrence of these three simultaneous phenomena make it difficult to establish a distinct case for each of the three.

This presentation is an attempt to cast new light upon the problems of hemodynamics by applying current rheological principles to the flow of blood. It is realized that the anticoagulant blood systems do not necessarily correspond to the circulating blood. However, for the interpretation of certain circulatory disorders we feel that the students of hemodynamics should be aware of probable relationships which adequately characterize the anomalous flow properties of blood.

#### SUMMARY

1 A modified falling ball viscometer (rolling ball viscometer) for blood and other humors is presented. It is capable of easily measuring flow properties at several stresses, as is required to define satisfactorily the properties of anomalous flow systems. At high shearing stresses, apparent specific viscosity values of  $2.5 \pm$  are observed, corresponding to  $2.2 \pm 0.2$  reported as possible with the biological viscometer of Whittaker and Winton.

2 Previous references to the anomalous flow properties of blood were verified. It was demonstrated that these systems conform to the Bingham concept of anomalous flow. To define completely the flow properties of such systems it is necessary to make determinations at at least two shearing stresses, preferably more. Data are reported for the pseudoviscosity and yield value, the latter being possibly the most specific property of the three bloods studied.

3 Heparin in increasing amounts tended to decrease the apparent viscosity, pseudoviscosity, and yield value of blood. Similar increases of heparin also reduced the viscosities of the serum and plasma.

4 The ratio of the apparent viscosity of blood and its plasma was found to be

reasonably constant as reported by Trevan. However, as the apparent viscosity is a function of the shearing stress, it is believed that the relationship for the calculations of corpuscular concentrations, such as the Whittaker and Winton modification of the Hatschek formula, is specific for the instrument and conditions of tests by which it was determined.

5 Heparinized blood was found to exhibit thixotropy, dilatancy, and age-hardening phenomena.

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# INCREASED IRRITABILITY IN NITELLA DUE TO GUANIDINE

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Guanidine<sup>1</sup> produces striking effects on animals which find a parallel in *Nitella*. This is the more interesting since these effects in animals have been attributed to nervous activity involving the action of acetylcholine.

The experiments on *Nitella* were undertaken because it appeared that loss of excitability and of the potassium effect (ability to distinguish electrically between  $K^+$  and  $Na^+$ ) in distilled water was due to the washing out of a substance which was called for convenience *R*. In some cases excitability remained after the potassium effect was lost and it was then found that the action current could restore the potassium effect.<sup>2</sup> Since the action current presumably carried cations from the sap into the surface it appeared possible that *R* contained organic cations. Accordingly experiments were made with guanidine since it can supply such cations, even at high pH, as it is a strong organic base.

The parallel phenomena may be considered under two heads.

**1 Restoration of Excitability**—In cases of myasthenia gravis the muscles may lose their excitability so that the patient is unable to move even an eyelid. Dosage with guanidine may then produce great improvement of the excitability and use of the muscles.<sup>3</sup>

When cells of *Nitella* have lost their excitability as the result of exposure to distilled water it may be restored by guanidine; this is also true of the potassium effect.<sup>4</sup>

**2 Hyperexcitability**—Guanidine may cause fibrillary tremors and tonic contractions of skeletal muscles.<sup>5,6</sup> The threshold for electrical stimulation necessary to cause muscular contraction when the stimulus is applied to the nerve is lowered.<sup>7</sup> It has been suggested as the result of experiments on dogs that guanidine sensitizes the muscle to the action of acetylcholine.<sup>7</sup> A similar suggestion comes from studies on myasthenia gravis.<sup>8</sup>

<sup>1</sup> For convenience this term will be used to include guanidine, methyl guanidine, and di-methyl guanidine.

<sup>2</sup> Osterhout, W J V, and Hill, S E. *J Gen Physiol* 1934-35 18, 681.

<sup>3</sup> Minot, A. S. Dodd, K. and Riven, S. S. *Science* 1938 87, 348 and the literature there given.

<sup>4</sup> Osterhout, W J V. *J Gen Physiol* 1940-41 24, 7.

<sup>5</sup> Paton, D. N., and Findlay, L., *Quart J Exp Physiol*, 1916 10, 315.

<sup>6</sup> Frank, E., Stern, R. and Nothmann, M., *Z ges exp Med*, 1921, 24, 341.

<sup>7</sup> Frank, E., Nothmann, M. and Guttmann, E., *Arch. ges Physiol*, 1923 201, 569.

Guanidine also produces hyperexcitability in *Nitella*. This is evident since (a) the threshold of electrical excitability<sup>8</sup> may be lowered and (b) a single electrical stimulus may produce a long series of quick action currents,<sup>9</sup> which is perhaps analogous to the fibrillary tremors of muscle under the action of guanidine.

There is no reason to suspect that acetylcholine plays a rôle in *Nitella* since the application of acetylcholine produces no change in P.D. and does not act as a stimulus. It would therefore seem that in *Nitella* guanidine sensitizes the protoplasm directly to the electrical stimulus.

A typical response to a single stimulus in an untreated cell is seen in Fig. 1.<sup>10</sup> But when cells are treated with guanidine a stimulus instead of producing a single response may give the result seen in Fig. 2. To explain such curves let us consider the role of  $K^+$  in producing P.D.<sup>11</sup> The normal outwardly directed (positive) P.D. is presumably due chiefly to the outwardly directed concentration gradient<sup>12</sup> of  $K^+$  across the inner protoplasmic surface, called for convenience *Y* (the aqueous part of the protoplasm may be called *W* and the outer non-aqueous surface *X*).

This gradient presumably disappears<sup>13</sup> when  $K^+$  moves outward into *W* as the result of the stimulus.

<sup>8</sup> The electrical stimulus consisted of 100 to 500 mv. D.C. applied at a distance of 1 cm. or more from the spot recorded, as described in previous papers.

<sup>9</sup> Such series of action currents are frequently propagated along the cell. They are more apt to occur in cells that have been kept a long time in the laboratory.

<sup>10</sup> The cells, after being freed from neighboring cells, stood in the laboratory at  $15^\circ \pm 1^\circ C$  in Solution A (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87) for several days. They belonged to Lot B (cf. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, 24, 312).

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 541). Temperature about 20-26°C. Regarding the amplifier see the reference just cited.

There was no indication of injury in these experiments.

<sup>11</sup> Strictly speaking they may be said to depend on the movement of ions in general but the effect of  $K^+$  is so predominant that we may, for convenience, confine the discussion to it.

<sup>12</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, 18, 215.

<sup>13</sup> Cole and Curtis (Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, 1938-39, 22, 37) have shown that the outwardly directed P.D. begins to fall off before the increase in the conductivity of the protoplasm begins. This might be brought about in a variety of ways, e.g. by a decrease in the value of  $\mu_K - r_{Cl}$ , as occurs, for example, in *Valonia* under the influence of various organic substances involving no loss of resistance; such substances might be produced as the result of stimulation. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, 20, 13, *J. Cell and Comp. Physiol.*, 1941, 18, 129.

Under normal conditions  $K^+$  presumably moves out through  $\lambda$ , producing the initial rise in the curve (spike) seen in Fig 1. When the outwardly moving

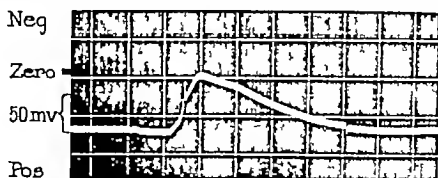


FIG 1 Action curve in an untreated cell recorded between two points  $D$  and  $F$  both in contact with Solution A (owing to a block the curve is monophasic)

The cell was freed from neighboring cells and kept in Solution A at  $15 \pm 1^\circ C$  for 50 days. The record was made at  $22^\circ C$ .

Heavy time marks 5 seconds apart

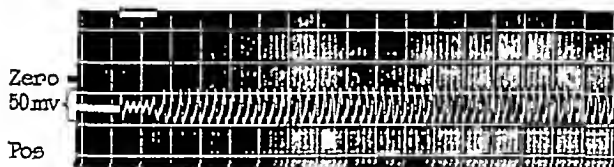


FIG 2 Result of a stimulation after an exposure of 3 hours to 0.01 M guanidine hydrochloride. During the passage of the stimulating current (shown by the white signal line above) the amplitude of the responses remained small but it subsequently increased and became steady (for about 7 minutes) and then the responses ceased abruptly. These quick action currents were propagated along the cell.

The cell was freed from neighboring cells and kept in Solution A for 50 days at  $15 \pm 1^\circ C$ . It was then kept at  $22^\circ C$  for a short time and exposed to the reagent at this temperature.

The spot recorded was in contact with Solution A and was connected through the galvanometer to a spot at the end of the cell in contact with Solution A saturated with chloroform which kept its p.d. constant approximately at zero and made the record monophasic.

Heavy time marks 5 seconds apart

$K^+$  reaches the outer non aqueous surface layer  $\lambda$  it sets up an outwardly directed concentration gradient of  $K^+$  and an outwardly directed p.d. causing a fall of the curve. This is reversed as  $K^+$  reaches the outside of  $\lambda$  and thus destroys the outwardly directed concentration gradient of  $K^+$  across  $\lambda$ .



Hence the curve rises. Then recovery sets in: this consists in restoring to the sap the  $K^+$  which has moved outward during the action current.

Experimental evidence favoring this explanation is found in cases where the sensitivity of  $X$  to  $K^+$  can be altered. Thus in *Nitella* we can make  $X$  insensitive to  $K^+$  by leaching with distilled water. We then find, as expected, only one peak in the action curve.<sup>14</sup> Conversely in *Chara* where  $X$  is normally insensitive to  $K^+$  and the action curve has but one peak we find, as expected, a double peak when  $X$  is made sensitive to  $K^+$  by means of guanidine.<sup>15</sup>

It may be added that in *Valonia*<sup>12</sup> the movement of  $K^+$  appears to produce effects resembling those seen in the action curve of *Nitella*.

This discussion applies to the normal procedure in which  $K^+$  is supposed to move outward into the cellulose wall or the external solution. Recovery is then relatively slow since it involves the return to the sap of the  $K^+$  which has moved out.<sup>16</sup> But if  $K^+$  moves outward only a very short distance, i.e. just outside  $X$ , it is evident that recovery could be much quicker. In that case the curve would have but one peak since  $K^+$  would not reach<sup>17</sup>  $X$ . We might then get such curves as are seen in Fig. 2. Whether the penetration of guanidine could accomplish this is an interesting question.

The action curves occurring under the influence of guanidine show interesting features.<sup>18</sup> Among these are the following:

1. In many cases bursts of action currents alternated with periods of rest (Figs. 3 and 4) (this is also seen in some untreated cells<sup>19</sup>). Similar phenomena have been observed in untreated nerve, e.g. by Adrian<sup>20</sup> and by Hoagland.<sup>21</sup>

2. The action curve may seem to go below the base line which records the

<sup>14</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1939-40, **23**, 743.

<sup>15</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1940-41, **24**, 9.

<sup>16</sup> This movement of  $K^+$  is presumably due to the forces which in the resting state of the cell cause  $K^+$  to move from the external solution to the sap.

<sup>17</sup> If the curve did not drop abruptly after reaching the apex of the spike we should not have a double peak. This abrupt fall of the curve can occur only if the outwardly moving  $K^+$  reaches  $X$  in the form of a fairly sharp moving boundary. This might be interfered with by protoplasmic motion which is usually present in *Nitella*.

If  $H$  already contains much  $K$  or guanidine ion the movement of  $K$  into  $H$  may not greatly increase the outwardly directed P.D. across  $X$  and hence may not cause much drop in the curve after the apex of the spike. In that case the first peak will be inconspicuous or lacking.

<sup>18</sup> Some of these have also been observed under the influence of NaCl or in cells which have stood for a long time in the laboratory in Solution A. Regarding these see Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 377, 1938-39, **22**, 91. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934-35, **18**, 499.

<sup>19</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934-35, **18**, 512 (Fig. 15).

<sup>20</sup> Adrian, E. D., *The Basis of Sensation*. London, Christophers, 1928.

<sup>21</sup> Hoagland, H., *J. Gen. Physiol.*, 1932-33, **16**, 695, 715.

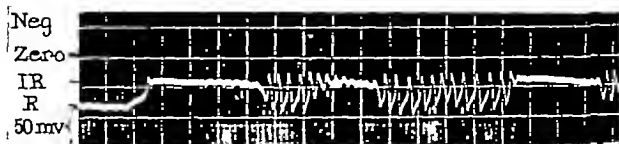


FIG 3 At the start the curve (*R*) shows the usual (complete) resting value of the P.D. then a spontaneous action curve is seen. No recovery occurs for about 20 seconds and during this interval the level of the curve may be called the incomplete resting stage (*IR*). It might be mistaken for the complete resting stage if we had not seen the rise of the curve: the subsequent quick action curves might then be erroneously regarded as carrying the curve below the complete resting stage (i.e. making the P.D. more positive than in the normal resting condition).

The record was made between two points *C* and *F* both in contact with Solution A (owing to a block the record is monophasic). The activity was spontaneous i.e. no stimulus was applied.

The cell was exposed for 117 minutes to 0.01 M guanidine hydrochloride at pH 5.

The cell was freed from neighboring cells and kept in Solution A for 30 days at  $15 \pm 1^\circ\text{C}$ . The record was made at  $22^\circ\text{C}$ .

Heavy time marks 5 seconds apart.

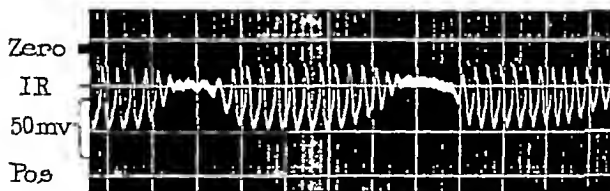


FIG 4 Shows a regular wax and wane of the amplitude of the action curve in a cell exposed for 4 hours to 0.01 M guanidine hydrochloride at pH 7. The record was made between two points *D* and *F*. *D* was in contact with Solution A and *F* was in contact with 0.01 M KCl which kept the P.D. constant approximately at zero and made the record monophasic.

Before and after the wax and wane phase the curve is at an incomplete resting stage (*IR*) as in Fig. 3. The activity was spontaneous i.e. no stimulus was applied.

The cell was freed from neighboring cells and kept in Solution A for 50 days at  $15 \pm 1^\circ\text{C}$ . The record was made at  $22^\circ\text{C}$ .

Heavy time marks 5 seconds apart.

P.D. of the cell at rest but it is probable that in such cases the base line does not represent the true resting potential. We may distinguish between the resting potential *R* found after complete recovery and the incomplete resting potential

$IR$ , found after incomplete recovery (*i.e.* when some of the  $K^+$  which moves from the sap into II during the action fails to go back into the sap during recovery and remains in II to set up an outwardly directed  $P.D.$  across X) For

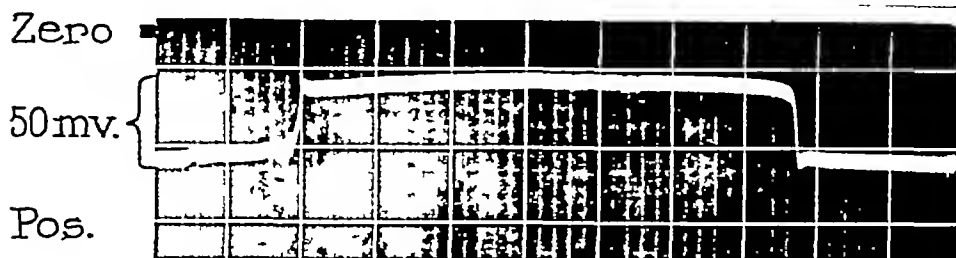


FIG 5 Shows a "square topped" action curve in a cell exposed for 140 minutes to 0.01 M guanidine hydrochloride at pH 7. The action curve is recorded between two points, *E* and *F*, both in contact with Solution A (owing to a block the action curve is monophasic). The activity was "spontaneous," *i.e.*, no stimulus was applied.

The cell was freed from neighboring cells and kept in Solution A for 30 days at  $15 \pm 1^\circ C$ . The record was made at  $22^\circ C$ .

Heavy time marks 5 seconds apart.



FIG 6 Transition to "square topped" action curve in a cell exposed for 61 minutes to 0.01 M guanidine hydrochloride at pH 7. The record was made between a spot *D* in contact with Solution A and another spot *F* in contact with 0.01 M KCl which kept the  $P.D.$  constant approximately at zero and made the record monophasic. The activity was spontaneous *i.e.* no stimulus was applied.

The cell was freed from neighboring cells and kept for 30 days in Solution A at  $15 \pm 1^\circ C$ . The record was made at  $22^\circ C$ .

Heavy time marks 5 seconds apart.

example in Fig 3 we see at the start a base line which may be regarded as representing the complete resting potential  $R$ . But later we see an incomplete resting potential  $IR$ . When recovery becomes more complete the curve goes below the  $IR$  level <sup>2</sup>.

<sup>2</sup> Oterhout, W. J. V. and Hill, S. E., *J. Gen. Physiol.*, 1934-35, 18, 499; Hill, S. E. and Oterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 91.

3 Action curves not going to zero The observed P.D. in the resting state is presumably due chiefly to the concentration gradient of  $K^+$  across I and when this disappears during the action the P.D. vanishes and the curve goes to

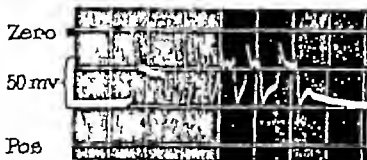


FIG 7 Transition to square topped action curves in a cell exposed for 61 minutes to 0.01 M guanidine hydrochloride at pH 7 The record was made between two points *D* and *F* *D* was in contact with Solution A and *F* with Solution A saturated with chloroform which kept the P.D. at *F* constant approximately at zero and made the record monophasic The activity was spontaneous i.e. no stimulus was applied

The cell was freed from neighboring cell and kept in Solution A for 30 days at  $15 \pm 1^\circ C$  The record was made at  $22^\circ C$

Heavy time marks 5 seconds apart



FIG 8 Transition to square topped action curve in a cell exposed for 53 minutes to 0.01 M guanidine hydrochloride at pH 7.0 The record was made between two points *D* and *F* with Solution A at *D* and Solution A saturated with chloroform at *F* which kept the P.D. at *F* constant approximately at zero and made the record monophasic The activity was spontaneous i.e. no stimulus was applied.

The cell was freed from neighboring cells and kept in Solution A for 30 days at  $15 \pm 1^\circ C$  The record was made at  $22^\circ C$

Heavy time marks 5 seconds apart

zero unless at the end of the upward movement (spike) of the curve some effective cations ( $K^+$ ,  $Na^+$  or guanidine ions<sup>22</sup>) remain in II to set up an out

<sup>22</sup> The apparent mobility of the guanidine ion in the outer protoplasmic surface exceeds that of  $Na^+$  and this may be true of the non myelinated nerve of the spider crab since according to Wilbrandt (Wilbrandt W *J Gen Physiol* 1936-37 20, 519) the guanidine ion acts somewhat like  $K^+$

wardly directed concentration gradient across  $X$ . Then there will be an outwardly directed P.D. across  $X$  and the curve will not go to zero. Several of the figures show this condition.<sup>22</sup>

4 Wax and wane in the amplitude of the action curves (Fig. 4). From what has just been said we may infer that a rise in the level of the apices of successive spikes indicates a decrease<sup>23</sup> in the concentration of effective ions ( $K^+$ ,  $Na^+$ , and guanidine ions) in  $W$  at the end of the spike. When the apices fall to lower and lower levels the opposite is indicated.

The presence of such ions in  $W$  at the end of the downward movement of the curve would prevent complete recovery and a decrease in such ions would make recovery more complete. Hence the increasing vertical amplitude in Fig. 4 might be explained as indicating a decrease and the wane as indicating an increase in the concentration of such ions in  $W$ .

5 Recovery is sometimes sudden, giving "square topped"<sup>24</sup> action curves as in Fig. 5.

It has been suggested<sup>25</sup> that recovery involves two operations, (a) the return from  $W$  to the sap of the  $K^+$  which comes out of the sap during the action and (b) the healing of breaks in  $I$  (such breaks may account for some of the increase in permeability which accompanies the action). If (a) occurs before (b) no recovery will occur until (b) is complete. The latter (healing of breaks)<sup>26</sup> might occur suddenly which can hardly be the case with (a). Various "transitions" to "square topped" action curves are seen in Figs. 6, 7, and 8.

It may be added that great variation in the response to the action of guanidine was observed and in some cases no response was obtained.

## DISCUSSION

In animals guanidine lowers the E.M.F. necessary to produce stimulation and sets up trains of action currents. These effects are also seen in *Mitella* but here there appears to be no reason for involving the action of acetylcholine as has been done with animals. When we apply acetylcholine to *Mitella* there is little or no effect and there is no evidence that it plays any part in stimulation. Even in animals it is doubtful whether guanidine acts by affecting sensitivity.

<sup>22</sup> See Osterhout, W. J. V., and Hill, S. E., Some ways to control bioelectrical behavior, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1936, 4, 47 (Fig. 3).

<sup>23</sup> Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 91. Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 417. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938-39, 22, 115.

<sup>24</sup> The production and healing of "breaks" due to surface tension may be sudden, as indicated by experiments with oily films. Cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, 11, 684; Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, 18, 221.

to acetylcholine. It has been suggested that guanidine affects potassium metabolism.<sup>27</sup>

Guanidine may produce such effects to some extent by increasing the conductivity of the aqueous layer of the protoplasm<sup>28</sup> (IV) as previously suggested in connection with the action of NaCl<sup>29</sup> in producing long trains of quick action currents. If we may judge by experiments on *Valonia*<sup>30</sup> it penetrates readily.

The shapes of the action curves might also be accounted for to some extent by the increased conductivity of IV as discussed in a previous paper in connection with NaCl<sup>31</sup>. It may be noted that according to Fühner<sup>31</sup> guanidine acts somewhat like NaCl on frog muscle.

The increased conductivity of the aqueous protoplasmic layer IV would permit the same current density to be attained with a lowered value of the applied E.M.F.

In addition guanidine may have specific effects as when it restores irritability and the potassium effect in *Nitella*.<sup>32</sup>

#### SUMMARY

Guanidine applied to *Nitella* may lower the threshold of E.M.F. required to produce electrical stimulation and may give rise to trains of action currents. Its effect thus appears to be somewhat similar to that observed in animals.

Rapid action currents are produced as well as "square topped" action curves and transitional forms.

These effects may be due in part to increased protoplasmic conductivity produced by the penetration of guanidine.

<sup>27</sup> Thompson V, and Tice, A., *J Pharm and Exp Therap*, 1941, 73, 455

<sup>28</sup> The resistance of the protoplasmic layer as measured is chiefly due to V and F and hence might not be much affected by guanidine.

<sup>29</sup> Hill, S E, and Osterhout, W J V, *J Gen Physiol*, 1938-39, 22, 91

<sup>30</sup> Osterhout W J V, Damon, E B, and Jacques, A G, *J Gen Physiol*, 1927-28, 11, 193

<sup>31</sup> Fühner, H. *Arch. exp Path u Pharm*, 1908, 58, 1

<sup>32</sup> Guanidine is able to denature proteins of Greenstein J P, *J Biol Chem*, 1939, 130, 519. Mirsky, A E, *J Gen Physiol*, 1940-41, 24, 709



# ALLOCATION OF ELECTRICAL RESPONSES FROM THE COMPOUND EYE OF GRASSHOPPERS\*

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## INTRODUCTION

The possible sites of origin of the electrical responses recorded from arthropod compound eyes have frequently been discussed in connection with the significance of these responses, but, except in *Limulus*, there is little experimental evidence concerning the definite allocation of these potentials. Hartline (1935) suggested that the electroretinogram of insects possibly originates in two places, the sensory membrane and the optic ganglion. This suggestion was supported by the investigations of Adrian (1937), who recorded a slow negative wave which apparently originated in the optic ganglion of *Dytiscus*, and of Roeder (1940), who noted that an electrical change of undetermined wave form could be produced in a grasshopper eye after excision of the optic ganglion. Bernhard (1942) demonstrated that most of the ERG of *Dytiscus* originates in the eye. He concluded that the ganglion does not contribute a slow wave to the ERG and, furthermore, that the slow wave Adrian (1937) recorded from the ganglion really originated in the eye. These conclusions of Bernhard (1942) on *Dytiscus* are not in agreement with preliminary reports by the present authors on *Trimerotropis* (Jahn and Wulff, 1941, 1941a).

The site of origin of the high frequency oscillations recorded from the compound eyes of insects is also of interest. Roeder (1939) suggested that in grasshoppers the oscillations originated in the cerebral ganglia. Jahn and Crescitelli (1940, 1941), Crescitelli and Jahn (1942), and Roeder (1940) then demonstrated that the oscillations persisted after the brain was removed and that the optic ganglion was probably the site of origin. This conclusion was also reached by Bernhard (1942) for *Dytiscus*.

The purpose of the present paper is to allocate the origin of certain components of the electroretinogram and of the electrical oscillations recorded from grasshopper eyes.

## Material and Methods

Two species of grasshoppers, *Trimerotropis cistrina* and *T. maritima*, were used in these experiments. In order to obtain the normal ERG and the electrical oscillations

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the animals were prepared in the following manner. Black paraffin chambers were built around the corneas of both eyes and fitted with glass end plates, one of which was rendered opaque to light by black paraffin. The tops were left open, and the chambers were filled with salt solution. The animals were then mounted in the electrode chamber, and electrical contact was made between reservoirs, which contained salt solution and non-polarizable electrodes, and the chambers about the eyes. One eye was il-

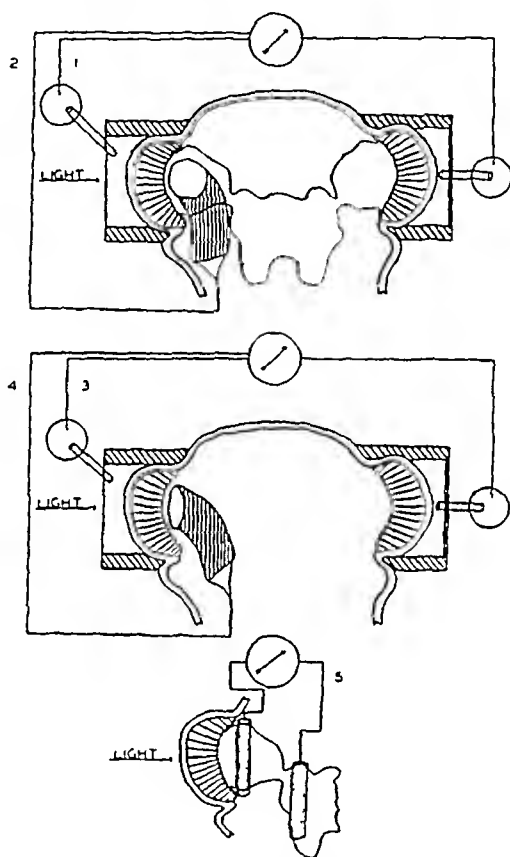


FIG 1 Diagrams illustrating electrode positions and electrical circuits. In the preparation involving leads 3 and 4 it is understood that the blood of the grasshopper completed the electrical circuit between the two eyes. The numbers on each circuit refer to the leads described in the text in full detail.

luminated for a controlled length of time with an intensity of 10,000 foot-candles, which will be referred to as unit intensity. The electrical responses were amplified by a variable-time-constant high-gain amplifier, and recorded on sensitized paper by means of a cathode ray oscillograph. For the slow potentials the amplifier was operated at a low gain and a long time-constant (2.9 seconds), for the faster oscillations the amplifier was operated at high gain and short time-constant (0.1 sec.).

The records presented in this paper were obtained with a variety of electrode positions, and in some cases the optic and cerebral ganglia were removed. The various lead combinations are diagrammed in Fig 1 and were as follows. Lead 1, input elec-

trode connected to illuminated cornea ground electrode to non illuminated cornea animal intact or with exoskeleton of front of head removed so that the ganglia could be easily removed later. Control experiments demonstrated that removal of part of the exoskeleton of the head did not affect the form or magnitude of the electroretinogram. Lead 2, input electrode (Ag AgCl or bare metal insulated to tip) placed on the optic ganglion ground electrode on non illuminated cornea. Lead 3 same as lead 1 except that the optic and cerebral ganglia were removed by simply lifting the ganglia out with a pair of forceps. Histological examinations of these preparations were made and are described below. Lead 4, animal deganglionated input electrode on back of eye in position formerly occupied by the optic ganglion ground electrode on non illuminated cornea. Lead 5 isolated eye and ganglion preparation, input electrode on ganglion ground electrode on the optic nerve or the crushed cerebral ganglia. In no case was the animal connected to ground except through the ground electrode.

## RESULTS

### *I Allocation of the Origin of the Electroretinogram*

*A The Electroretinogram of the Normal Animal (Lead 1)*—The wave form of the electrical response of the normal *Trimerotropis* eye to a half-second light stimulus of unit intensity varies with the state of light adaptation. The response of the maximally dark adapted eye is a negative (downward) wave consisting of a rapid *b*-wave<sup>1</sup> with a latent period of about 10 msec. a *c* wave which is a maintained potential, and a downward spike, the *d* wave (row 1 Fig. 2). The *d*-wave ends with a return to the base line.

Upon light adaptation this response changes in several ways. (1) The *b*-wave and the *d* wave spikes apparently decrease in size, this is caused by (2) an increase in the magnitude of the *c* wave. The magnitude of the *c* wave first undergoes a decrease and then a considerable increase (row 1, Fig. 2).

These responses of the eye of *Trimerotropis* differ from those of *Melanoplus differentialis* described previously in that the *b*- and *d* waves are present in the dark adapted eye. In *M. differentialis* these waves appear only under conditions of light adaptation (Jahn and Crescitelli, 1938). On the basis of the three-component theory (Granit, 1933) these differences are explainable by assuming slightly different magnitudes or wave forms for the components.

*B The Electroretinogram of the Deganglionated<sup>2</sup> Animal (Lead 3)*—The wave form of the electroretinogram of the deganglionated eye is characteristically and constantly different from that of the normal eye in several respects. (1) The *b*-wave spike is modified into a blunt peak which ends in a

<sup>1</sup> The terminology applied to parts of the typical vertebrate electroretinogram have been carried over and applied to the arthropod electroretinogram but a fundamental similarity between the two is not necessarily implied.

<sup>2</sup> The operative procedure in the removal of the optic ganglion is described below in the section on morphology.

slow return to the level of the *c*-wave, (2) the *d*-wave spike and all evidence of the *d*-wave are absent, (row 3, Fig 2), (3) the *c*-wave usually has a somewhat greater upward slope than that of the normal ERG (intermediate and light-adapted conditions), (4) the effect of light adaptation is much reduced and results only in a slight increase in size of the *c*-wave

Fig 3 shows the result of one of a series of experiments in which the ERGs of the same animal before and after deganglionation were compared Record

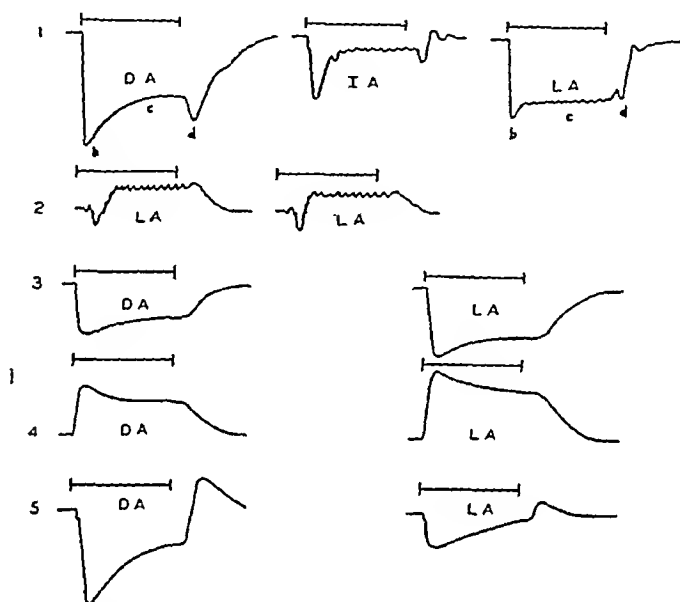


FIG 2 Copies of typical responses obtained from normal and deganglionated compound grasshopper eyes and optic ganglia The number at left of each row of records corresponds to the number of a lead in Fig 1 All exposures were one-half second DA denotes dark-adapted, IA, intermediate-adapted, and LA, light-adapted In row 1 the DA, IA, and LA records are the first, tenth, and one-hundredth responses from a repetitive series Time-constant 2.9 seconds Downward deflection denotes negativity of left lead in Fig 1

II A consists of normal ERGs obtained from an animal before deganglionation Record II B was obtained from the same animal after deganglionation Similar experiments were performed on a number of animals The principal differences between records II A and II B have been observed in every case studied and strongly suggest a contribution from the optic ganglion to the normal ERG

*C Normal and Deganglionated Electretinograms Recorded from the Back of the Eye (Leads 2 and 4)*—With the input electrode placed on the optic ganglion and bathed by the blood of the grasshopper (lead 2) an inverted and modified form of the normal ERG is obtained (row 2, Fig 2) There is an alteration of wave form, but by far the most significant difference is a greatly

increased latent period. The latency is about three times as great as the same response recorded from the corneal surface (approximately 30 msec. vs. approximately 10 msec). The peak of the potential with lead 2 is considerably later in appearance than the peak of the *b*-wave with leads 1, 3 or 4.

This increase in the length of the latent period becomes significant only when it is compared with a similar recording of the deganglionated ERG (lead 4). The response obtained with the deganglionated animal is the inverted form of the deganglionated ERG recorded from the corneal surface (lead 3) and there is no increase in latency and very little distortion of wave form (row 4 Fig. 2).

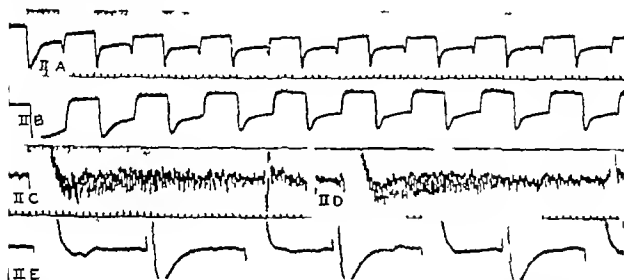


FIG. 3 Typical responses of the normal and deganglionated compound eye of grasshoppers. II A normal responses to half second repetitive exposures to 10 000 foot candles. II B responses of deganglionated animal to same stimulus as in II A. II C II D oscillatory response of normal animal. II E record showing lack of oscillatory response in deganglionated animal. All records were taken from the same eye of one animal. Records II A and II B were taken with a time constant of 2.9 seconds and at intermediate amplifier gain. Records II C, II D and II E were taken with a 0.1 second time constant and at maximum amplifier gain. Time signal 100 msec. Refer to text for further details.

These results would seem to indicate that with lead 2 the optic ganglion was the source of a potential which interacted with the potential from the eye, thus giving rise to the observed differences, and that with lead 4 only one potential source—that of the eye—existed. (The possibility that two components, one positive and one negative, occur in the eye will be discussed later.)

In a few cases the deganglionated eye gave a simple deflection in the positive direction comparable to a square wave which was maintained during illumination. In these cases the basement membrane suffered obvious injury and the source and significance of this potential difference remains uncertain.

*D. Slow Potentials Recorded from the Isolated Eye Ganglion Preparation (Lead 5)*—The response of the ganglion to a half-second light stimulus is a

diphasic response, the major portion of which is a slow negative wave (row 5, Fig 2) The wave form remains constant with light adaptation, but the magnitude decreases In all cases the optic ganglion first became electrically negative to the optic nerve and cerebral ganglia The latent period of this ganglion potential is about 28 msec This is longer than the latent period with leads 1 and 2, but not quite so long as that of lead 3

The wave form of these responses, particularly the return toward the base line during illumination and the positivity with a return to the base line upon cessation of illumination, might be taken upon superficial examination to be caused by amplifier distortion (too short a time-constant) This possibility, however, is eliminated by the fact that responses of similar length and magnitude from the corneal surfaces of the eye (records II A and II B, Fig 3, rows 1, 2, 3, 4, Fig 2) show no such distortion and were obtained with the same amplifier characteristics Therefore, the peculiar wave form of these slow potentials may be considered as the result of spreading of the potential change over the surface of the ganglion Apparently because of the short length of the optic nerve and the lack of a myelin sheath the ground electrode in lead 5 is not "indifferent" but is in electrical contact with the active region of the ganglion in such a way that the leads are "diphasic" There is no reason to assume that the spreading of potential over the ganglion is much different in lead 1 (except, perhaps, for a slowing of the spread because of possible damage to the ganglia), but the lead in that case is apparently "monophasic" The curve of potential change at any one point on the ganglion probably simulates a "square" wave Since with half-second exposures the "on" effect is greater than the "off" effect (row 5, Fig 2), this square wave probably declines in magnitude

## *II Allocation of the Electrical Oscillations*

The electrical oscillations used in these experiments fall into the class of intermediate adaptation rhythms, as defined by Crescitelli and Jahn (1942) This rhythm is present when the eye is partially light-adapted but disappears with both extreme light and dark adaptation In these experiments it was observed that the intermediate adaptation rhythm could be elicited by a stimulus only when the optic ganglion behind the illuminated eye was present and uninjured (records II C and II D, Fig 3) When the optic ganglion was surgically removed electrical oscillations were never obtained (record II E, Fig 3) The oscillations are sometimes of greater magnitude when the recording electrode is placed on certain parts of the ganglion

In the isolated eye ganglion preparations previously described the slow potential was always present, but the electrical oscillations were never recorded This failure was attributed to possible slight injury of the ganglion Roeder (1939) encountered a comparable phenomenon when he failed to obtain oscillations after severing the optic nerve Later (Roeder, 1940) he pointed out that this absence of oscillations was caused by slight injury of the ganglion

### III Morphological Observations

Surgical removal of the optic ganglion, which is closely attached to the back of the eye, was facilitated by a natural tendency of the ganglion to separate easily from the eye close to the basement membrane. This resulted in almost complete removal of the neurone layers of the ganglion with minimal injury to the sensory elements of the eye.

The optic ganglion is composed of three neuropiles or synaptic areas, one, the lamina ganglionaris, almost immediately behind the basement membrane and two larger groups, the medulla externa and medulla interna, toward the center of the head. The neurocytes are on the periphery of the ganglion and between the neuropiles. The ganglion is covered by a neurilemma except on the side adjacent to the basement membrane. In the deganglionated animals the two median neuropiles (medulla externa and medulla interna) and all of the neurilemma were completely removed. In all cases the lamina ganglionaris was considerably damaged and partly removed and the basal retinal pigment was clearly visible from the back of the eye. The only neurocytes which were not removed were those few which were between the lamina ganglionaris and the basement membrane. As will be discussed below it is highly improbable that these neurocytes contributed to the recorded potential. In some few cases the basement membrane seemed to be considerably injured, and in these cases the recorded action potential was a simple maintained deflection with reversed polarity.

### DISCUSSION

#### *Summary of Evidence for Allocation of Potentials*

The conclusions drawn from the series of experiments which have been described are (1) that the optic ganglion is the site of origin of electrical oscillations and (2) that the slow potential of the optic ganglion is a component of the electroretinogram of the grasshopper. The various evidences for these conclusions will be presented.

#### *I Evidence for the Conclusion that the Optic Ganglion Is the Site of Origin of the Electrical Oscillations*

It has been clearly demonstrated (Jahn and Crescitelli, 1940, 1941, Crescitelli and Jahn, 1942, and Roeder, 1940) that the cerebral ganglia are not in any way concerned with the high frequency oscillations recorded from the eyes of grasshoppers. In the present series of experiments we have demonstrated that the electrical oscillations are present when the optic ganglion behind the eye is present and uninjured, and absent whenever the ganglion has been mutilated or removed.

These observed facts point clearly to the optic ganglion as the source of high frequency oscillations, a conclusion corroborated by the fact that these

oscillations are of greater magnitude when recorded from certain parts of the intact optic ganglion

## *II Evidence for the Conclusion That the Slow Potential of the Optic Ganglion Contributes to the Grasshopper ERG*

*A Direct Evidence*—A comparison of the responses obtained from the corneal surfaces of the normal and deganglionated grasshopper reveals distinct and constant differences which have been observed in every case studied. It is concluded, therefore, that removal of the optic ganglion is directly responsible for the changes produced in the ERG. This reasoning leads to the logical assumption that the ERG of the normal animal is an algebraic sum of two potentials, one originating at the sensory membrane and the second originating at the optic ganglion.

That the optic ganglion actually is the source of a slow potential is amply illustrated by the records taken with the isolated eye ganglion preparation (lead 5). As explained above, this change of potential probably simulates a square wave of declining amplitude. The results of Adrian (1937) on *Dytiscus* demonstrate that in this animal the optic ganglion potential is a negative wave which is maintained during illumination. On the basis of other work on ganglia and on the vertebrate central nervous system a more or less maintained negative wave is the type of potential change which might be expected.

On the basis of the above observations, it is possible to reconstruct the normal ERG by algebraic summation of the ERG from the deganglionated animal (*i.e.*, the potential from the sensory membrane) and the potential from the optic ganglion. In this reconstruction it is necessary to account for the following characteristics of the normal ERG: (1) the *b*-wave spike, (2) the *d*-wave spike, and (3) the increase in magnitude of the *c*-wave upon light adaptation. All these three characteristics are partially or totally absent in the electroretinogram from deganglionated animals.

During illumination the back of the compound eye becomes electrically positive to the front of the eye, and the optic ganglion becomes negative to the optic nerve. Therefore, it might seem likely that when measurements are made across both the eye and the ganglion, the negativity of the ganglion should oppose the positivity of the back of the eye, and the recorded potential should be the sum of the potentials of these two sources. However, the observed potential approximates the difference rather than the sum of these two potentials, and this fact makes it seem likely that the fibers going into the ganglion make electrical contact with the inside of the ganglion (*i.e.*, with the positive area) and that the negativity of the outer side of the ganglion is recorded in opposition to the potential from the sensory membrane. The algebraic summation of these potentials is shown diagrammatically in Fig. 4.

On this basis we can account for the above three characteristics of the normal

ERG as follows (1) Since the latency of the optic ganglion response is greater than that of the ERG of the deganglionated animal, the initial blunt negative spike of the former will react with the initial peak of the ERG to produce the *b*-wave spike of the ERG of the normal animal, (2) the response of the optic ganglion is terminated by a rapid change in the positive direction, and it is this change which adds (because of its location) to the declining negative potential from the sensory membrane to give rise to the *d* wave spike of the ERG of the normal animal. (3) The magnitude of the two peaks recorded from the isolated ganglion and presumably of the maintained potential of the ganglion decreases with light adaptation (row 5, Fig 2). This decrease in the ganglion

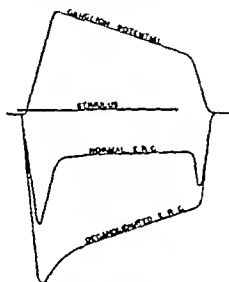


FIG 4 Diagrammatic representation of the algebraic summation of potentials from the sensory membrane (deganglionated ERG) and optic ganglion to produce the normal ERG. The potentials above the base line are positive; those below the base line are negative. Refer to text for further details.

potential will account for the increase in magnitude of the *c* wave upon light adaptation in the ERG of the normal animal. In Fig 4 it can be noted that the summation of the two potential changes results in a slight inflection on the front of the *b*-wave of the normal ERG. This inflection is usually visible in original records of the normal ERG, especially in slightly light-adapted animals, and is barely perceptible in record II A of Fig 3. This inflection was never observed in records from deganglionated animals.

This close similarity between the response of the optic ganglion and the arbitrary summation of the responses of the normal and the deganglionated eyes indicates the probability that such a process of algebraic summation actually occurs. The exact wave forms of the responses may be somewhat different from those of Fig 4 because of slight condenser distortion (time-constant of 2.9 sec.) but the basic contours must be very similar to those presented.

In this discussion the possibility that the neurocytes remaining near the



basement membrane after deganglionation may contribute to the potential has not been considered. It is unknown whether or not these neurones were still active after removal of the ganglion. However, since the ganglion potential as ordinarily measured is either a difference between the inside and outside of the ganglion or between ganglionic axons and neurocytes it seems highly probable that when most of the ganglion and all of the neurilemma have been removed, any potential developed by the remaining ganglion cells would be shunted in such a manner that it would be undetectable with the leads used.

*B Indirect Evidence*—It has been pointed out that the normal electroretinogram, when recorded from the back of the eye, exhibits a marked increase in the latent period, whereas the deganglionated electroretinogram, when recorded from a similar electrode position, exhibits no such discrepancy in latency.

These results may be explained on the assumption of the existence of two potential sources in the normal animal, one source being the sensory membrane, the other the optic ganglion. Since these fields would exist almost simultaneously an electrode placed anywhere within the superimposed areas of these potential fields would record the algebraic summation at that particular point. The wave form and latency resulting from this algebraic summation would change with the location of the recording electrode.

When one of the potential sources, the optic ganglion, is removed, the electrode records only the one potential, which may be the inverted form of the response obtained from the corneal surface of the eye of the deganglionated animal.

This line of reasoning coincides in every respect with the experimental observations and therefore affords good indirect evidence for support of the conclusion that the slow potential of the optic ganglion exists and is summed algebraically with the slow potential of the sensory membrane.

#### *Other Attempts to Allocate the Potential*

The only other serious attempt to allocate the origin of the insect ERG is that of Bernhard (1942). Bernhard used isolated eye and eye ganglion preparations of *Dytiscus marginalis*. Recording electrodes were placed on the cornea and at various places along the optic and cerebral ganglia and on both sides of the isolated eye. When the ganglia were removed it was found that the oscillations were no longer recorded and that the ERG resembled a smooth square wave. Cocainization resulted in a similar ERG recorded between the cornea and cerebral ganglia. Recording from the back of the eye and the cerebral ganglia of the cocaine preparation resulted in an electrical change similar to and of the *same* electrical sign (negative) as recording from the cornea. This potential decreased in magnitude as the active recording electrode was moved along the optic ganglion toward the cerebrum. Bernhard concluded that this potential was the electrotonic spread of the ERG. Previously,

Adrian (1937) had described this same potential as a ganglion potential, but Bernhard (1942) did not agree with this interpretation.

On the basis of Bernhard's interpretation, the front part of the eye upon illumination becomes more negative than the back part of the eye, and the whole eye (or at least both surfaces of the eye) must become negative to the cerebral ganglia. The first conclusion is amply supported by Bernhard's records of *Dytiscus* and our own records of *Trimerotropis* and unpublished records on other grasshoppers. However, the second conclusion is based only on records taken from the optic ganglion and cerebrum of *Dytiscus*. Records comparable to those from lead 4 (of present paper) are not readily obtainable from isolated preparations, but they have been obtained from deganglionated specimens of *Trimerotropis*. Our records demonstrate conclusively that with lead 4 the back of the eye gives a positive potential. Since lead 5 gives a negative potential it seems logical to assume that, at least in *Trimerotropis*, the ganglion gives rise to a negative wave, and that the potential measured on the ganglion is not the electrotonic spread of the (positive) eye potential. In *Dytiscus* this problem warrants further investigation.

#### *Vertebrate vs. Invertebrate Components*

The theory has been presented herewith that the normal electroretinogram of *Trimerotropis* is formed by the summation of a ganglion potential and a potential from the sensory membrane. This is essentially similar to the theories of two or three components which are supposedly summed in order to give the vertebrate ERG (Kohlrusch, 1931, Granit, 1938). At present we have no basis for assuming which, if any, of the components of the vertebrate ERG are comparable to the two which are herewith proposed for the grasshopper. In previous publications (e.g., Jahn and Crescitelli, 1938, 1940, Jahn and Wulff, 1942) the nomenclature of the vertebrate ERG has been adopted, but it has also been pointed out that the cause of this adoption has been a matter of convenience rather than a conviction that the two wave forms are directly comparable. The same caution should be observed in the case of the components.

However, on the tentative assumption that the components proposed herewith might be directly comparable to those proposed for the vertebrate eye, a comparison may be made. The so called negative component (P III of Granit) is in the direction opposite from that of the normal ERG and accounts for the *a* and *d*-waves. In the grasshopper, according to the present evidence, the ganglion potential assumes a similar position and accounts for the sharpness of the *b*-wave and for the *d* wave.<sup>3</sup> If we assume that the ganglion potential is

<sup>3</sup> In the moth it has been demonstrated (Jahn and Crescitelli 1939) that the *a*-wave, depending upon the state of adaptation may precede the *b*-wave may be a notch on the *b*-wave or may be preceded by the *b*-wave in such a manner as to increase the descending slope of the *b*-wave. In the moth the components must differ considerably

strictly comparable to the negative component (P III) of vertebrates, and that P III is a process in the ganglionic neurones, we must then conclude that the activity of these ganglionic cells is much less susceptible to the action of toxic agents than is that of the site of origin of components I and II, for component III is always the last to disappear under the action of ether (Granit, 1933), low temperature (Nikiforowsky, 1912), and potassium (Therman, 1938). It seems, *a priori*, as if the ganglion potential should be quite sensitive to these reagents, and one might expect component III, on the basis of its low temperature coefficient, to be developed more or less directly by the photochemical process in the sense cells. For that reason it does not seem possible to conclude that the ganglion potential is strictly comparable to the negative component (P III) of vertebrates. It is possible that P III may be comparable to the negative potential obtained from grasshopper eyes with an injured basement membrane. However, further analysis of possible homologies must await future investigation.

Bernhard (1942) concluded that there were two components in the ERG of *Dyliscus*, one which was an index of receptor activity and one which resulted from light adaptation. The two components herewith proposed for *Trimerotropis* are not the same as those described by Bernhard. We have made no attempt to separate the deganglionated eye potential into components. The fact that the back of the deganglionated eye becomes positive may be considered as evidence of a "dipole"—producing mechanism or it may be taken to indicate that the ERG consists of two processes, one of which makes the back of the eye positive (*cf.* Therman, 1940). This problem is beyond the scope of the present paper.

The ERG of the deganglionated *Trimerotropis* eye presents certain similarities to the ERG of other arthropod eyes. The ERG of *Limulus* in response to a stimulus of one-half second or longer is a simple deflection which reaches a maximum rapidly and then subsides to a low potential which is maintained during illumination (Hartline, 1928, 1935). This type of ERG differs from that of the deganglionated *Trimerotropis* eye principally in the relative magnitudes of the maximum and the maintained potentials. Since the optic ganglion of *Limulus* is separated from the eye and does not participate in the ERG, one might expect a similarity between the response of *Limulus* and that of the deganglionated insect eye. In the crayfish, the ERG is very similar to that of *Limulus* (unpublished observations by Jahn and Crescitelli). The crayfish optic ganglion does not cover the back of the eye and is separated from it by a constriction,

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in wave form from those of the grasshopper, especially since both *a*-waves and sharp *b*-waves (even multiple *b*-waves) may be present. However, it seems permissible to assume that the ganglion contributes a negative component comparable to that of *Trimerotropis*.

and it seems as if summation of ganglionic and sensory membrane potentials does not occur

The wave form of the ERG of *Melanoplus* is considerably simpler than that of *Trimerotropis*. It seems quite probable that the principal differences between the ERGs of these animals may be caused by the geometrical relationships of the eye and optic ganglion. In relation to the size of the eye the optic ganglion of *Trimerotropis* is relatively much larger than in *Melanoplus*, and it seems probable that the relatively larger ganglion may prevent shunting in such a way that more of the ganglion potential is summed with sensory membrane potential to produce the recorded ERG. This idea is supported by the fact that in *Melanoplus* the wave form of the ERG is only slightly affected by removal of the ganglion (Jahn and Wulff, unpublished observations). It also seems possible that the geometrical configuration of the eye and ganglion of *Dytiscus* may explain Bernhard's observation that removal of the ganglion does not change the major wave form. In the case of animals which undergo a diurnal rhythm in the wave form of the ERG (Jahn and Crescitelli, 1940, Jahn and Wulff, 1942) such a simple explanation is not sufficient to account for the differences between the day and night type of responses.

#### SUMMARY

1 The effect of extirpation of the optic ganglion on the ERG and on electrical oscillations recorded from the compound eye was determined

2 Extirpation of the optic ganglion prevents the occurrence of oscillations, and it is concluded that they originate in the ganglion.

3 Extirpation of the optic ganglion changes the wave form of the ERG. The sharpness of the *b*-wave is decreased, the relative magnitude of the *c* wave is increased, and the *d* wave is obliterated. These changes can be explained by assuming that the ERG is the algebraic sum of two potential changes, one in the compound eye, and another of opposite sign in the ganglion. This assumption is supported by data from a number of experiments in which the electrode positions were varied.

4 The explanation of the present data (which indicates two sites of origin of the ERG) is similar to the three-component theory which accounts for the complex wave form of the vertebrate ERG.

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# STUDIES ON THE METABOLISM OF AUTOTROPHIC BACTERIA\*

## I THE RESPIRATION OF *THIOBACILLUS THIOOXIDANS* ON SULFUR

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### INTRODUCTION

The strictly autotrophic bacteria are defined as microorganisms which grow only in the presence of a specific inorganic nutrient from whose oxidation they derive their energy and which cover all of their carbon needs from carbon dioxide. They are of physiological interest for three principal reasons. First, they permit an experimental test of many of the deductions of comparative physiology and biochemistry in that they are examples of life conducted under conditions intolerable for most other forms. The principal deduction derivable from such comparative studies seems to be that there is a basic unity in all living processes, that there are certain things which always take place wherever life exists. The evidence for this basic unity in living processes has been receiving considerable attention (*cf* van Niel (1940), Peterson (1941)). The autotrophic bacteria, by their very nature, offer the possibility of an experimental approach to the problems which arise from the concept of a basic unity in all life processes. Second, the process of chemosynthesis (defined as the formation of all cell materials from  $\text{CO}_2$  in the dark using chemical energy derived from the oxidation of the specific nutrient) is thought to be closely related to the process of photosynthesis so that knowledge of its mechanism might aid in the solution of the latter problem. Third, since the autotrophic bacteria are able to live in a purely inorganic environment it has been supposed (*cf* Werkman (1939)) that they might be the surviving examples of the first forms of life. As such, a study of their metabolism would be of interest since they might contain primitive types of metabolic processes. The papers of this series are concerned with studies on one of the strict autotrophic bacteria, *Thiobacillus thiooxidans* and are divided into three parts (1) the mechanism of sulfur oxidation as revealed by respiration studies, (2) the manner in which  $\text{CO}_2$  is synthesized, and (3) the mechanism of energy exchange in chemosynthesis. This paper is concerned with the mechanism of sulfur oxidation.

The organism studied *Thiobacillus thiooxidans*, oxidizes sulfur (or thio-sulfate) to sulfuric acid obtaining thereby the only energy which will permit it to grow. Carbon dioxide is the only carbon source available for growth. It

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is not influenced by, nor does it apparently utilize, most types of organic materials, sugars, for example. It will grow in an extremely acid solution containing more than 5 per cent sulfuric acid. The strain used in these studies was discovered by Waksman and Joffe (1922) and was obtained through the kindness of Dr. Waksman. It has been studied physiologically by Waksman and Starkey (1922, 1923) and Starkey (1925 *a*, 1925 *b*). Methods of preparing suspensions and details of growth have been previously described (Umbreit, *et al*, 1942). Briefly these consist of growth for several days under pure culture conditions on a medium containing no organic materials, only a few mineral salts, and sulfur. After the growth period any remaining sulfur is filtered off and the suspension is centrifuged in a Sharples supercentrifuge. The cells are washed in distilled water and kept in distilled water suspension.

Previous studies from this laboratory have justified the claim that the study of autotrophic bacteria is of importance from the viewpoint of comparative physiology. It was shown (Umbreit *et al*, 1942) that the oxidation of sulfur (which is insoluble in the medium employed for growth) actually takes place within the bacterial cell, the sulfur being dissolved in a fat globule located at the terminal ends of the cell. This supports the generalization that no organism can obtain energy from oxidation not carried within its cell or at the cell surface. Further, in spite of the fact that organic materials are apparently not utilized, the organism possesses an organic metabolism utilizing the storage products formed during sulfur oxidation (Vogler, 1942). Cells of this autotrophic bacterium grown on a purely inorganic medium contained the following vitamins (O'Kane, 1942): thiamin, pyridoxine, biotin, nicotinic acid, pantothenic acid, and riboflavin. This observation lends support to the generalization that these materials are of universal significance and that lack of a "growth factor" requirement represents the ability of the organism to synthesize the factor involved. It therefore appears that in spite of certain distinctly unusual properties of this autotroph, there exist certain points of marked similarity with the metabolism of other types of cells, and in this sense the data support the concept of a basic unity in all living organisms.

Respiration studies applied to autotrophic bacteria are not entirely satisfactory since they supply only one, or at most only a few, estimates of activity and as such are not entirely conclusive. But they are of importance since in this way it is possible to separate growth reactions from those concerned with the metabolism of the resting cell. They provide the basic background for other methods of studying metabolism and are essential for the study of chemosynthesis. Much of our present knowledge of autotrophic bacteria has been obtained by these methods (Boemke, 1939), (Meyerhof, 1916 *a*, 1916 *b*, 1917), (Vogler, 1942), (Waksman and Starkey, 1922). Inasmuch as a necessary condition for sulfur oxidation is a direct contact between the bacteria cell and the sulfur particle (Vogler and Umbreit, 1941) certain modifications

in the methods which can be applied to this organism were required. These are described in the following section.

### *Methods*

Oxygen uptake in the presence of sulfur was measured at 28°C. in the Warburg apparatus with KOH in the center cup *s. e.*, in the absence of all but traces of CO<sub>2</sub>. The sulfur employed in these experiments was supplied in the form of a water suspension containing about 200 mg sulfur per ml which was used so that the sulfur was always in excess. Commercial "flowers of sulfur" is highly water repellent and does not form suitable suspensions. Colloidal sulfur from H<sub>2</sub>S and SO<sub>2</sub> or from thiosulfate is satisfactory but an easy way to obtain large quantities of sulfur suitable for suspension in water is the use of the sulfur which remains in the cultures at harvest. Such sulfur is filtered from the cultures and suspended in  $\pi/1$  KOH which dissolves any organisms that may be still attached to it. After about an hour in the KOH it is washed repeatedly with distilled water until free from traces of KOH. The sulfur thus obtained is free from organic materials and is composed of rather small particles. It can be kept in water suspension (from which it settles fairly rapidly) and is readily pipetted out into equal samples.

The changes in oxygen uptake which occur when sulfur is mixed with a suspension of the organism in the Warburg flask have been described by Vogler (1942) who employs the same technique as used in these studies. The bacterial suspension, the appropriate buffer (usually  $\pi/60$  KH<sub>2</sub>PO<sub>4</sub>), and a suspension of finely divided sulfur are mixed and incubated for several hours. Immediately before use the pH is adjusted to the proper value with as little disturbance as possible. Aliquots containing not more than 20 micrograms bacterial nitrogen per ml are then used in the Warburg determinations. One factor which influences the rate of oxygen uptake is the rate of shaking of the flasks since this tends to disturb the sulfur bacteria contact. Shaking is only necessary when the rate of oxidation is very high since here it is necessary to permit oxygen penetration. Some compromise must be made between oxygen diffusion and the disturbance of cell contact. This is done empirically. The rate of shaking is standardized at an optimum value and is held constant for the entire series of experiments. Nitrogen was determined on the bacterial suspensions by the micro-Kjeldahl procedure described by Johnson (1941). Other specialized methods are described in the text when necessary. The phosphate concentration influences the rate of sulfur oxidation but that rate is constant for any given phosphate level. When materials are tipped in from the side arms of the flasks, these are dissolved in the same phosphate buffer so that the total phosphate concentration does not change.

The handling of suspensions without sulfur is entirely comparable to the procedures used in studies of heterotrophic bacteria. Suspensions without sulfur may be held at refrigerator temperatures for several weeks (in distilled water, saline, or phosphate) but they should not be too concentrated. About 200 micrograms bacterial nitrogen per ml. are satisfactory.

### *Factors Influencing Respiration*

*Temperature*—Sulfur oxidation was found to proceed over a temperature range from 18–45°C. The influence of temperature on sulfur oxidation was



measured by determining the oxygen uptake of a series of aliquots at 28°C over 2 successive hours to be certain that the sulfur oxidation in all had reached a constant rate. The flasks were then transferred to other water baths kept at different temperatures and the oxygen uptake compared with controls left at 28°C. The manometers in all baths were shaken on the same rocking device. After all temperatures except 48°C, respiration was unimpaired when the flasks were returned to 28°C. The data obtained are given in Table I. The energy of activation, " $\mu$ ," calculated from these data is 19,000 calories (between 28–30°C) and 7,500 calories (between 18–28°C). These values have a rather large error attached to them and are of interest only in that they are of the same magnitude as those found for a variety of other respiratory processes. Crozier (1924) called attention to the fact that the  $\mu$  values of various bio-

TABLE I  
*The Effect of Temperature on Sulfur Oxidation by Thiobacillus thiooxidans*

Temperature °C	No. of samples	Respiration 28°C = 100	Standard deviation
7	2	7	—
18	4	69	5.5
28	8	100	—
33	8	189	47
38	5	286	32
43	3	286	38
48	2	177	—

logical reactions clustered around certain figures, *i.e.*, 8,000, 12,000, and 18,000. The observation that sulfur has about the same temperature relationship as have other biological oxidation processes points toward a relationship of the respiratory processes in autotrophic and in heterotrophic organisms.

*Acidity*—The influence of pH upon the activities of *Thiobacillus thiooxidans* is recorded in Fig. 1 which illustrates the respiration obtained with resting cell suspensions. The curves show that above a pH of 5 there is a marked inhibition of sulfur oxidation (and thiosulfate oxidation) but there is little effect upon the endogenous respiration in the absence of sulfur. There is a remarkable correlation between inhibition of respiration at these pH values and the amount of bound CO<sub>2</sub> in the medium. However, since sulfur oxidation is continued in the Warburg flask in the presence of KOH in the center well, lack of free CO<sub>2</sub> cannot account for the inhibition of respiration at pH values where carbon dioxide is mainly present as carbonate or bicarbonate. It is of interest, however, that the organism is adapted to just that range of H<sup>+</sup> ion concentration in which free CO<sub>2</sub> is dissolved in the medium as a gas.

At pH values higher than 8, lysis occurs and the cells dissolve. The endogenous respiration is, of course, destroyed by this treatment.

At pH values lower than 5, two types of response to alterations in pH are obtained depending upon whether the oxygen uptake is measured after the organisms have been in contact with sulfur for some time, or immediately after the sulfur has been added. The first type of response (that of organisms which have been in contact with sulfur for some time) is represented in Fig. 1 by cells which have been in contact with sulfur for 4 hours. This curve shows

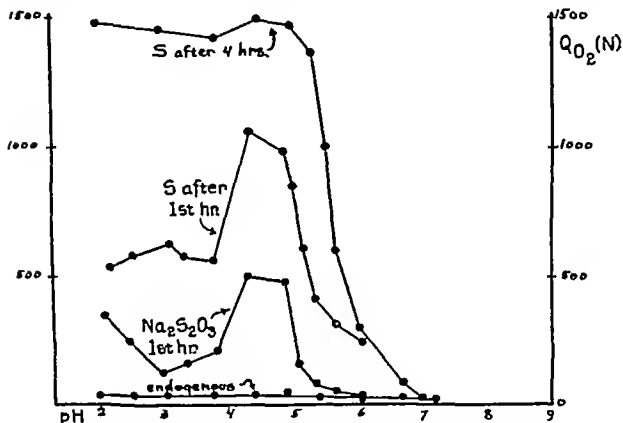


FIG. 1 The influence of pH upon the respiration of *Thiobacillus thiooxidans*

that the oxidation of sulfur is not appreciably influenced by changes in pH between the limits of pH 4.8-2.0. If, however, sulfur (or thiosulfate) is added to the suspension and the oxygen uptake measured immediately a different shape curve is obtained. The shape of this curve would suggest that the phenomenon is due to the electrical charges on the organism and the sulfur. Since the phenomenon occurs only during the period in which direct contact is being established there seems to be no doubt that the effect is on the rate of formation of the contact and not upon sulfur oxidation as such. It is of considerable interest that sodium thiosulfate has the same type of curve as sulfur. Since it is soluble, one would expect that its oxidation would not be dependent upon sulfur bacteria contact. That it suggests that

thiosulfate is actually taken within the cell in the form of sulfur. Growth studies reported by Waksman and Starkey (1923) show a pH relationship closely similar to the respiration curves obtained after sulfur-bacteria contact has been established. These curves of growth and respiration differ from most pH curves obtained with bacteria in that they do not have marked optima. This suggests that the internal pH of the cell is virtually independent of the external pH below a pH of 4.5.

*Pressure of Oxygen*—Growth studies indicated that variation of the  $pO_2$  between 10 and 30 per cent had very little effect upon the rate of formation of sulfate (Vogler and Umbreit, 1941). Fig. 2 summarizes the data obtained with respect to the effect of  $pO_2$  on the rate of sulfur and thiosulfate oxidation by resting cells. The curves labeled I, II, III, and IV represent successive half-hour intervals. In the lower graph it may be noted that the effect of increased oxygen pressure is to increase the rate of sulfur oxidation but this increase is not marked. Reduced oxygen pressure likewise has little effect. One point of interest is the tendency to increased rates of sulfur oxidation at lower pressures of oxygen. This increased rate tends to become greater with time. These data serve to emphasize that the rate of sulfur oxidation is not nearly as sensitive to  $pO_2$  as the literature implies and as might be expected if the oxidation of sulfur were a simple chemical reaction. The effect of  $pCO_2$  is very complex and will be considered in later papers.

*Organic Substrates*—In accordance with the reports of previous investigators it was found that, as a rule, organic materials had no effect upon respiration. No organic material yet found can replace sulfur or  $CO_2$  for the growth of *Thiobacillus thiooxidans*. Fructose, galactose, cellobiose, glucose, trehalose, dulcitol, glycogen, starch, arabinose, xylose, gluconic acid, and glycerophosphate had no effect upon either sulfur oxidation or respiration in the absence of sulfur. However, pyruvic, succinic, fumaric, malic, and oxalacetic acids appeared to stimulate respiration in the absence of sulfur. Citric acid was variable. Of these, pyruvic and lactic acids ( $M/150$ ) inhibited sulfur oxidation quite consistently while succinic and fumaric acid occasionally inhibited. None of these could serve as a source of energy for growth (in the absence of sulfur). The effect of these materials is being studied further. One unusual effect has been noted, pure crystalline riboflavin inhibits growth on sulfur at concentrations of 10 micrograms per ml and also inhibits sulfur oxidation by resting cells, yet riboflavin is synthesized by the cell growing in an inorganic medium.

*Inhibitors*—A wide variety of inhibitors have been studied for their effect upon sulfur oxidation, on respiration in the absence of sulfur, and for their effect on growth. The data are recorded in Table II. In the case of sulfur oxidation the inhibitor was tipped in from the side arm after a constant rate of sulfur oxidation had been established. The inhibitors were suspended in

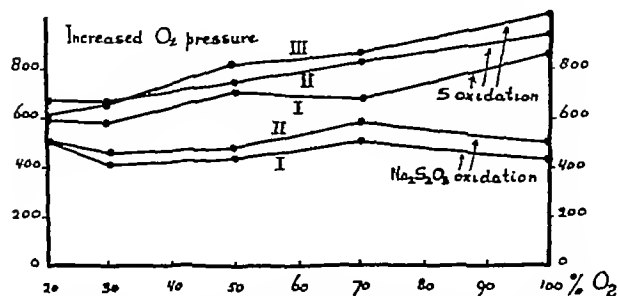
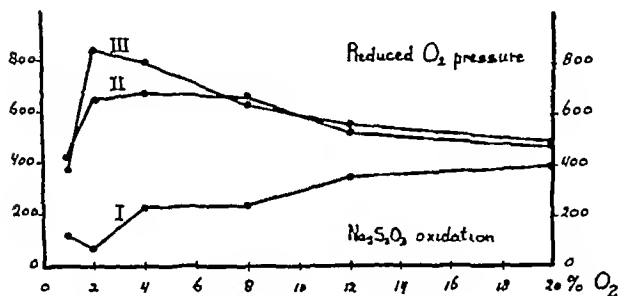
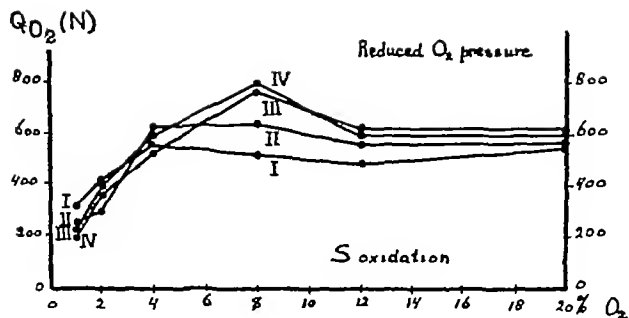


FIG 2 The influence of  $pO_2$  upon sulfur and thiosulfate oxidation by *Thiobacillus thiooxidans*

phosphate ( $M/30$ ) so that there was no change in phosphate concentration upon the addition of the inhibitor. All experiments were run at pH 4.6–4.8 in  $M/30$   $KH_2PO_4$ .

At the present state of our knowledge of the action of these inhibitors on intact cells, the data contained in Table II can be considered merely as a statement of experimental results. For example, the point at which indole acts upon respiratory processes in intact cells is not known. Its peculiar action on this organism (inhibition of growth at  $10^{-6}M$ , sulfur oxidation at  $10^{-3}M$ , and respiration in the absence of sulfur at  $10^{-2}M$ ) can merely be given as an experimental finding and no interpretation can as yet be given.

Of the entire group of inhibitors studied only two inhibited the respiration in the absence of sulfur more than sulfur oxidation. These (semicarbazide, hydrazine) are characterized by the common possession of a  $-N-NH_2$  group. Urethane and sodium malonate had little effect upon any of the processes studied. The action of sodium pyrophosphate, which inhibits growth at concentrations having no effect upon respiration seems to be related to its rather slow diffusion into the cell. Sodium arsenite inhibits growth at much lower concentrations than respiration, but these data alone could not be regarded as evidence of a phosphorylation since the locus of inhibition by arsenite in intact cells is not well established. Sodium fluoride gives extremely variable results, frequently a marked stimulation is noted. This may be followed by an inhibition at lower concentrations. This effect has been noted in several experiments but no explanation can be given upon the basis of data now available.

2,4-Dinitrophenol (DNP) causes 100 per cent inhibition when its concentration reaches a threshold value close to  $M/40,000$ . At lower concentrations it tends to stimulate respiration. These effects are better illustrated in Table III in which the concentration of this agent is increased by smaller steps. The definite stimulating effect at low concentrations of dinitrophenol cannot be ascribed to lack of penetration of the inhibitor into the cells at low concentrations. The more gradual effect on endogenous respiration, which becomes inhibited only at a 10 times greater concentration (and is still stimulated at  $M/40,000$ ) also indicates that diffusion phenomena are not the decisive factor in these experiments. Unfortunately the locus of the action of dinitrophenol is not known. That it does not "prevent assimilation" seems now fairly well established but beyond this point little is known of its action.

The inhibition of sulfur oxidation and to a lesser extent the endogenous respiration by potassium acid phthalate was unexpected inasmuch as phthalate buffers have been used quite generally in physiological studies. This effect, however, is not confined to the autotroph, respiration of *Escherichia coli* and *Staphylococcus aureus* was inhibited by  $M/40$  potassium acid phthalate. The respiration of *Bacillus subtilis* was not affected (all of these were respirations

TABLE II

*The Influence of Inhibitors on the Respiration and Growth of Th. thiooxidans*

Figures in parentheses indicate stimulation. Growth studies indicate growth (+) or no growth (-) in the presence of sulfur. No further changes were evident after the 2 week period recorded. Endogenous respiration means respiration in the absence of oxidizable sulfur. Inhibition recorded as per cent of respiration destroyed by addition of inhibitor. All studies at pH 4.6-4.8 in  $M/30$   $KH_2PO_4$ .

Molarity of inhibitor (final concentration)	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	Remarks
Sodium cyanide							
Sulfur oxidation		100	50	50	0	0	
Endogenous respiration		95	32	10	0	0	
Growth 1 wk.			-	-	-	-	
2 wks.			-	+	+	+	
Sodium azide							
Sulfur oxidation		92	86	60	5	0	See text
Endogenous respiration		0	0	0	0	0	
Growth 1 wk.		-	-	-	-	-	
2 wks.		-	-	+	-	-	
Sodium iodoacetate							
Sulfur oxidation		100	100	97	60		
Endogenous respiration			40	26	0		
Growth 1 wk.		-	-	-	-	-	
2 wks.		-	-	-	-	+	
Sodium fluoride							
Sulfur oxidation		93	10	(12)			See text
Endogenous respiration		37	(37)	27			
Growth 1 wk.	-	-	-	+	+	+	
2 wks.	-	-	-	+	+	+	
Sodium pyrophosphate							
Sulfur oxidation		0	0				See text
Endogenous respiration		0	-				
Growth 1 wk.		-	-	+	+	+	
2 wks.		-	-	+	+	+	
Sodium arsenite							
Sulfur oxidation	-	74	42	0			
Endogenous respiration	-	5	5				
Growth 1 wk.	-	-	-	-	-	+	
2 wks.	-	-	-	-	-	+	

TABLE II—*Continued*

Molarity of inhibitor (final concentration)	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	Remarks
Urethane							
Sulfur oxidation	35	0					
Endogenous respiration	0						
Growth 1 wk	+	+					— } at M/1 — }
2 wks	+	+					
2,4-Dinitrophenol							
Sulfur oxidation		100	100	100	18		See text
Endogenous respiration		100	92	71	26		
Growth 1 wk			—	—	—	+	
2 wks			—	—	—	+	
Phloridzin							
Sulfur oxidation			(10)				
Endogenous respiration			0				
Growth 1 wk			+	+			
2 wks			+	+			
Sodium malonate							
Sulfur oxidation	0	0					0 at M/1
Endogenous respiration	0	0					
Growth 1 wk	—	—	+	+	+	+	—
2 wks	—	—	+	+	+	+	
Indole							
Sulfur oxidation		74	15	0			See text
Endogenous respiration		48	0	0			
Growth 1 wk		—	—	—	—	—	
2 wks		—	—	—	—	—	
Semicarbazide							
Sulfur oxidation		(9)	(10)				
Endogenous respiration		34					
Growth 1 wk		—	—	+			
2 wks		—	+	+			
NH <sub>2</sub> OH (hydroxylamine)							
Sulfur oxidation	(14)	(13)	(19)				
Endogenous respiration	0	0	0				
Growth 1 wk	—	—	+				
2 wks	—	—	+				

TABLE II—*Concluded*

Molarity of inhibitor (final concentration)	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	Remarks
NH <sub>2</sub> CONH <sub>2</sub> (urea)							
Sulfur oxidation		10	6				
Endogenous respiration		0	0				
Growth 1 wk.	+	+	+				- } at M/1 - }
2 wks.	+	+	+				
NH <sub>2</sub> - NH <sub>2</sub> (hydrazine)							
Sulfur oxidation		(10)	(18)				
Endogenous respiration		40					
Growth 1 wk.		-	-	-	+	+	
2 wks.		-	-	-	+	+	
Hydroquinone							
Sulfur oxidation		95	28				
Endogenous respiration		35	(17)				
Growth 1 wk.		-	-	-	+	+	
2 wks.		-	-	+	+	+	
K-phthalate							
Sulfur oxidation		92	13	7			See text
Endogenous respiration		49	0	0			
Growth 1 wk.		-	-	-	-	+	
2 wks.		-	-	-	+	+	

in the absence of added substrate) It occurred to us that the phthalate inhibition might be related to its structural similarity to the dicarboxylic acids (particularly succinic) This was actually found to be the case,  $M/40$  phthalate inhibited succinate oxidation by a purified enzyme preparation 30 per cent The stimulating effect of pyruvic, succinic, malic, and fumaric acids upon the endogenous respiration of the antotroph could be removed entirely by the addition of  $M/40$  phthalate The inhibited rate of respiration in the presence of the organic acids was higher than the inhibited rate in their absence but not as high as the uninhibited respiration in the absence of organic acid

While the effects of sodium azide upon sulfur oxidation are quite consistent, the action on the endogenous respiration is somewhat variable Frequently there is no effect (as reported by Vogler, 1942), sometimes there is an inhibition, just as frequently there may be a stimulation The averages of several experiments show essentially no effect upon the endogenous respiration so



that this result has been recorded in the table. There seems to be an effect on both processes at higher concentrations ( $M/1$ )

Sodium cyanide inhibits sulfur oxidation and endogenous respiration (these experiments were done with cyanide in the KOH of the center cup since at a pH of 4.6, HCN would tend to distill into the KOH, cf Krebs, 1935). Carbon monoxide (80 per cent in 20 per cent oxygen) inhibits sulfur oxidation 50 per cent (not recorded in Table I). This inhibition is completely relieved by light and returns in the dark in the manner shown by Warburg (1927) to be characteristic of the iron respiratory systems.

Sodium iodoacetate inhibits sulfur oxidation more markedly than endogenous respiration.

TABLE III  
*Inhibition of Sulfur Oxidation by Dinitrophenol*

Concentration of dinitrophenol	Inhibition of sulfur oxidation
$M/640,000$	(40)*
$M/320,000$	(47)
$M/160,000$	12
$M/100,000$	18
$M/80,000$	44
$M/40,000$	96

\* Values in parentheses indicate stimulation

#### DISCUSSION

Among the effects reported here several are of further interest. The determination of the  $\mu$  values ("energy of activation"), while subject to considerable error yielded results comparable to those of heterotrophic respiratory systems and different from values which might be expected from the chemical reaction involving only the oxidation of sulfur. The  $pO_2$  had little effect upon the oxidation contrary to what would be expected if sulfur oxidation were a simple chemical reaction. Certain inhibitors (azide, cyanide, and carbon monoxide) point to the participation of a system very much like cytochrome in the oxidation of sulfur. It is of interest that Emoto (1933) reported the presence of cytochrome in a closely related sulfur-oxidizing bacterium. Cytochrome, however, is known to be an electron-transporting system, and oxygen is thought to enter the reaction only at the end of a relatively long series of electron transfers. While sulfur oxidation is undoubtedly a transfer of electrons it is difficult to see where the oxygen of the sulfate originates on this basis. One could visualize, and Bunker (1936) has pointed out, the process of sulfur oxidation as a hydration of sulfur followed by the removal of an electron from the hydrate to the cytochrome system. In such a visualization, the oxygen that

is respired does not enter into the sulfate molecule at all. Rather the oxygen which appears in the sulfate comes from water. Proof or disproof of such a theory must await the application of isotopic techniques. For example, if oxidation were to proceed in this manner in a solution containing water with heavy oxygen, the heavy oxygen should be found in the sulfate formed.

#### CONCLUSIONS

The data of this paper indicate that

1 The "energy of activation" ( $\mu$ ) of sulfur oxidation by the autotrophic bacterium, *Thiobacillus thiooxidans*, is similar to that of other respirations.

2 The pH of the menstruum does not influence the respiration on sulfur between the limits of pH 2 to 4.8 once contact between the bacterial cell and the sulfur particle has been established but it does influence the rate at which such contact occurs.

3 The  $pO_2$  has little effect upon the respiration of this organism.

4 Most organic materials have no detectable effect upon the respiration of *Thiobacillus thiooxidans*, but the organic acids of terminal respiration seem to stimulate the respiration in the absence of oxidizable sulfur and certain of them inhibit sulfur oxidation.

5 In so far as inhibitor studies on intact cells are trustworthy, sulfur oxidation goes through iron containing systems similar to cytochrome. It is possible that the oxygen contained in the sulfonic acid formed during sulfur oxidation is derived from the oxygen of the water.

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acid was placed. The whole system was equilibrated and when the  $\text{CO}_2$  was to be added, the vent was turned slightly which dislodged the sodium carbonate tube, allowing it to drop into the sulfuric acid and liberate its  $\text{CO}_2$ . This insures the addition of pure  $\text{CO}_2$ . (2) The second method of  $\text{CO}_2$  addition is as follows. The gas vents of the Dixon-Keilin flasks were connected with pressure tubing to a gas reservoir containing pure  $\text{CO}_2$ . The  $\text{CO}_2$  was flushed through the vents until the system could be considered to contain pure  $\text{CO}_2$ . The vents were then plugged into the side arms. This means of filling the vents did not always yield pure  $\text{CO}_2$  but blanks could be used to determine the actual amount of  $\text{CO}_2$  added to each flask. Pure  $\text{CO}_2$  could be insured, however, by evacuating the vents after they were plugged in the side arms and refilling with pure  $\text{CO}_2$ . Several evacuations and refillings are necessary. The  $\text{CO}_2$  is kept at a constant pressure in the reservoir (slightly higher than atmospheric pressure) by means of a water column and trap. Pressure tubing is used throughout to prevent the escape of  $\text{CO}_2$  through the rubber and to permit evacuation of the vents. When  $\text{CO}_2$  is to be added the vents are opened to the inside of the flask so that the  $\text{CO}_2$  enters.

The amount of  $\text{CO}_2$  added to the flasks by either method is easily calculated since during the short time the  $\text{CO}_2$  remains in the side arm no appreciable quantity dissolves in the solutions of the flask or is taken up by the organism. The amount of  $\text{CO}_2$  added ( $a$ ) is calculated from the equation

$$(1) \quad a = V_G \frac{273}{T} \frac{h_a}{P_0} \quad (\text{Symbols those of Dixon (1934)})$$

in which  $h_a$  is the increase in pressure read on the manometer immediately after the addition of  $\text{CO}_2$ . After the allotted time has expired the sulfuric solution containing the inhibitor (which is used in both methods) is tipped into the flask from the side arm. This stops further cell activities and also releases any possible bound  $\text{CO}_2$ . After this addition it is necessary to allow a little time for equilibration since the addition of sulfuric acid results in the formation of some heat (heat of dilution) and a slight contraction of the mixture (which is very close to the contraction observed when sulfuric acid is mixed with water). When equilibrium has been reached, the KOH is added to the center cup by turning the bottom stopcock of the Dixon-Keilin flask. The resultant change in pressure ( $h_b$ ) is read on the manometer after all the  $\text{CO}_2$  from the gas phase has been absorbed (which takes about an hour) and enables one to calculate the  $\text{CO}_2$  remaining in the atmosphere of the flask ( $b$ ) since

$$(2) \quad b = V_G \frac{273}{T} \frac{h_b}{P_0}$$

The difference  $a - b$  is the amount of  $\text{CO}_2$  which has disappeared from the gas phase. The portion of this which has dissolved in the medium does not enter into the reading  $b$ . It will, of course, eventually distill over into the KOH but the pressure change is caused only by the  $\text{CO}_2$  absorbed from the gas phase.

The amount of oxygen taken up can be determined by comparing the reading just before  $\text{CO}_2$  was added and after the  $\text{CO}_2$  has been removed. This value is corrected for the contraction which occurs on mixing the sulfuric acid with water and which

is measured in the blank flask. This gives a measure of the oxygen uptake which is independent of the  $\text{CO}_2$  measurement. It has the limitation however, that only the initial and end values of oxygen uptake are determined. Intermediate points may be obtained by the use of a series of replicate flasks stopped at different intervals.

The main problem is then to discriminate between the  $\text{CO}_2$  dissolved in the medium and that taken up by the cells. This was accomplished by running blank samples containing killed cells. Several methods of obtaining blank samples (as closely alike in composition to the living cell suspensions as possible) were employed. The principal methods employed were (1) heating of the suspension followed by cooling and shaking in air to equilibrate to atmospheric  $p\text{CO}_2$ , or (2) additions of  $\text{HgCl}_2$  (0.1 per cent) or iodoacetate (0.001 per cent) in sulfuric acid. The addition of these materials (or the heat treatment) removes the  $\text{CO}_2$  uptake due to living cells and leaves only that which dissolves in the medium or the cell substance. The observed  $\text{CO}_2$  uptake under these conditions is quantitatively the same with all methods of killing (or in the entire absence of cells) and hence is only due to  $\text{CO}_2$  solubility. This amount of dissolved  $\text{CO}_2$  could be correlated with calculations based on the determined  $\alpha$  value and the concentrations of  $\text{CO}_2$  in the atmosphere.

This type of calculation can be made as follows. If  $\alpha$  represents the number of milliliters of the gas dissolved in 1 ml. of the solution at a pressure of 1 atm. the amount of  $\text{CO}_2$  dissolved at  $1/n$  atmospheres will be  $1/n \alpha$  (Henry's law). At the point of equilibrium, the amount of  $\text{CO}_2$  dissolved in the solutions is equal to the amount soluble at the partial pressure of  $\text{CO}_2$  in the atmosphere at that point or if  $a$  is the amount of  $\text{CO}_2$  added and  $x$  is the amount dissolved, it can be easily shown that

$$(3) \quad x = \frac{\alpha V_f}{V_a + \alpha V_f} a$$

The  $\alpha$  values for each medium can be determined with this formula from the blank containing killed cells. The volume of  $\text{CO}_2$  dissolved in the fluid can thus be readily calculated for the exact experimental conditions employed and the  $\text{CO}_2$  fixed by the cells can be determined by correcting the total  $\text{CO}_2$  uptake observed by the amounts of  $\text{CO}_2$  dissolved.

From the same blank the concentration of  $\text{CO}_2$  in the laboratory atmosphere may be determined from the difference between the reading before the  $\text{CO}_2$  was added and the reading after all the  $\text{CO}_2$  had been absorbed by  $\text{KOH}$ . The  $\text{CO}_2$  concentration in the laboratory air varied from 0.04 to 0.07 per cent (corresponding to from 4 to 21  $\mu\text{l. CO}_2$  per flask). This is constantly recovered in blanks and is indicative of the accuracy of the method.

In calculating the amounts of  $\text{CO}_2$  dissolved in the medium during the course of an experiment from the amounts of  $\text{CO}_2$  present in the atmosphere of a flask at a given interval equation (3) is slightly modified to

$$(4) \quad x = \frac{\frac{\alpha V_f}{V_a + \alpha V_f} a}{1 - \frac{\alpha V_f}{V_a + \alpha V_f}} a$$

in which  $a_t$  represents the amounts of  $\text{CO}_2$  left in the atmosphere of the flask at time  $t$ , as calculated from the total amount present at the initial point less the total uptake plus the oxygen uptake over the time  $t$ . See Table II for such a calculation.

The addition of the necessary amounts of gases sometimes extends the pressure changes beyond those measurable directly on the manometer. A method to extend the manometer range is therefore useful. None seems to have been described in the literature. Such a method, however, is easily derived from the equality of

$$(5) \quad VP = RT \text{ or } V_1P_1 = V_2P_2 \text{ at constant } T$$

When the volume of gas  $V_G$  is increased  $x$  by changing the level of the manometer fluid in the closed arm over a height of  $e$  cm, the corresponding pressure  $P_0$  will be decreased by  $y$  so that

$$(6) \quad (V_G + x)(P_0 - y) = V_GP_0 \text{ or } V_Gy = x(P_0 - y)$$

Similarly, if the fluid in the closed arm be lowered  $f$  centimeters the corresponding volume change ( $z$ ) will cause a decrease  $y'$  so that

$$(7) \quad V_Gy' = z(P_0 - y')$$

Thus

$$(8) \quad y/y' = \frac{x(P_0 - y)}{z(P_0 - y')} = \frac{e(P_0 - y)}{f(P_0 - y')}$$

since  $x = r^2e$  and  $z = r^2f$

where  $r$  is radius of capillary tube of the manometer. But since  $P_0$  is large (10,000 mm of Brodie's solution) and  $y$  or  $y'$  are small, an accuracy of 1 per cent is possible if  $y$  and  $y'$  are not greater than 100 mm (10 cm) by considering  $P_0 - y = P_0 - y'$ , from which

$$(9) \quad y/y' = 1/f$$

But

$$(10) \quad y = y' - w$$

where  $w$  is the difference between the readings in the open arm of the manometer at  $e$  and at  $f$  or, therefore,

$$(11) \quad y = \frac{we}{f - e}$$

If  $f$  be chosen to equal  $2e$ , then  $y = w$

### *CO<sub>2</sub> Uptake in the Absence of Sulfur*

Carbon dioxide uptake during the oxidation of sulfur is an exceedingly complex phenomenon so that it is perhaps better to begin with the  $\text{CO}_2$  uptake by cells in the absence of sulfur. This was not expected inasmuch as the literature implies that  $\text{CO}_2$  uptake is limited to the period of sulfur oxidation. In fact, so rigidly has  $\text{CO}_2$  uptake been associated with growth of *Thiobacillus*

*thiooxidans* that, at least from evidence available until now, its uptake might be "growth bound". It is evident from the following sections in which  $\text{CO}_2$  uptake is observed in resting cells, that the  $\text{CO}_2$  fixation reaction is not "growth bound" but can occur in the absence of growth.  $\text{CO}_2$  uptake in the absence of sulfur was further surprising in that Vogler (1942) had shown that a characteristic of endogenous respiration was the production of  $\text{CO}_2$ . This phenomenon ( $\text{CO}_2$  liberation during endogenous respiration) was observed in suspensions from 14 day old cultures. In suspensions obtained from young cultures (about 7 days old), the endogenous oxygen uptake was much lower and  $\text{CO}_2$  was often not liberated. Instead, these suspensions were capable of taking up small but definite amounts of  $\text{CO}_2$  from the atmosphere. After  $\text{CO}_2$  had been taken up to saturation the suspensions again formed  $\text{CO}_2$  during endogenous respiration. After some study it became apparent that we were dealing with two types of suspensions with properties about as follows

'Young suspensions'—harvested 6-8 days after inoculation

Endogenous respiration low  $Q_{O_2}(N) = 4-10$

Sulfur oxidation high  $Q_{O_2}(N) = 2-3000$

$\text{CO}_2$  taken up during early stages of endogenous respiration.

'Old suspensions'—harvested after 12-14 days

Endogenous respiration high  $Q_{O_2}(N) = 20-40$

Sulfur oxidation low  $Q_{O_2}(N) = 2-400$

$\text{CO}_2$  released during endogenous respiration

Suspensions of either type may be held for several weeks at refrigerator temperatures but during this time they show some loss in activity. The "young" type of suspension may be converted into the older type by other means than aging, i.e., any method which causes the release of its energy stores. The old type of suspension may be converted into the young type by allowing it to oxidize sulfur for a short while. Only the young type of cell shows  $\text{CO}_2$  fixation in the absence of sulfur.

The data contained in Table I show  $\text{CO}_2$  fixation by young suspensions harvested after 7 days. This  $\text{CO}_2$  fixation is observed in the absence of sulfur. Considering first the blank containing cells killed with  $\text{HgCl}_2$  (flask 1), 680  $\mu\text{l}$  of  $\text{CO}_2$  were added at zero time. Of this amount, 52  $\mu\text{l}$  dissolved in the solution. At the end of 15 hours the addition of KOH absorbed 656  $\mu\text{l}$ . This amount plus the 52  $\mu\text{l}$  dissolved yields 708  $\mu\text{l}$  which is a recovery of the  $\text{CO}_2$  added plus the  $\text{CO}_2$  which was present in the air at the start of the experiment. This amount of  $\text{CO}_2$  originally present in the atmosphere of the flask is thus calculated as  $708 - 680 = 28 \mu\text{l}$  which corresponds to a concentration of  $\text{CO}_2$  in the atmosphere of 0.06 per cent (about that usually found in the laboratory air). From this value the amount of  $\text{CO}_2$  present in the other flasks may be calculated (Table I, column 2). Under these circumstances there was, of

course, a small amount of  $\text{CO}_2$  dissolved in the solution at the start of the experiment, but this amount was very small and does not exceed  $4 \mu\text{l}$  so that it can be neglected. In the case of flasks Nos 4 and 5, containing living cells, 573 and  $654 \mu\text{l}$  of  $\text{CO}_2$  were added respectively. At the end of 15 hours of respiration the addition of KOH caused an almost negligible absorption (No 4 =  $27 \mu\text{l}$ , No 5 =  $29 \mu\text{l}$ ) indicating that the  $\text{CO}_2$  added had somehow disappeared. The organisms did not convert the  $\text{CO}_2$  to bicarbonate ("bound"  $\text{CO}_2$ ) since the addition of acid with the inhibitor from the side arm, to stop the activities of the cells before KOH was added, did not liberate any gas. Nor can solution in the medium account for the  $\text{CO}_2$  removed since this uptake

TABLE I

*The Uptake of  $\text{CO}_2$  and Oxygen by Resting Cells of *Thiobacillus thiooxidans* in the Absence of Sulfur*

Flask contents	CO <sub>2</sub> in flasks		Total uptake CO <sub>2</sub> + O <sub>2</sub>								Residual CO <sub>2</sub> at 15 hrs absorbed in KOH		Total CO <sub>2</sub> taken up 15 hrs	O <sub>2</sub> taken up 15 hrs	CO <sub>2</sub> re covered
	Added at zero hr	From atmos phere	Time in min							Hrs					
			10	20	40	60	120	180	15		μl	per cent atm	μl	μl	μl
1 Blank, HgCl <sub>2</sub> killed cells	680	28	49	51	52	51	52	52	51	656	2.7	52	0	708	
2 2000 micrograms bacterial nitrogen	0	25	0	2	5	18	36	54	123	24.0	0.6	—	123	24	
3 As 2	0	26	1	2	6	18	37	55	139	26.0	0.6	—	139	26	
4 As 2	573	34	129	214	360	439	548	607	705	27.0	0.7	569	126	596	
5 As 2	654	23	174	283	445	527	609	645	771	29.0	0.8	647	124	676	
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	

would have stopped at a level where an equilibrium between the amounts of  $\text{CO}_2$  in the gas phase and the liquid phase was reached. In the case of cells treated in exactly the same way except killed with  $\text{HgCl}_2$  (flask No 1) this solubility amounted to only  $52 \mu\text{l}$ . The data leave no doubt, therefore, that the  $\text{CO}_2$  was actually fixed by the organisms since virtually all of the  $\text{CO}_2$  disappeared from the atmosphere. The final amounts of  $\text{CO}_2$  in the gas phase were found to be practically the same in all flasks, corresponding to a value close to the per cent of  $\text{CO}_2$  in the laboratory air.

From the data of Table I, one can calculate the amount of  $\text{CO}_2$  actually fixed at each interval measured, by correcting the total uptake observed in flasks 4 and 5 by the oxygen uptake observed in flasks 2 and 3 and for the  $\text{CO}_2$  dissolved in the medium. This value can be calculated from the  $\alpha$  value determined in flask 1 and the  $\text{pCO}_2$  at each point. These values are given in

Table II which illustrates the method of calculation and the results are plotted in Fig 1 which is more convenient for discussion

TABLE II  
Calculations from the Data of Table I

For flask 5 only

Time, min.	10	20	40	60	120	180	15 hrs.
Total uptake observed $\mu$ l	174	283	445	527	609	645	771
Oxygen uptake, $\mu$ l	1	2	6	18	36	54	124
CO <sub>2</sub> uptake $\mu$ l	173	281	439	509	573	592	647
CO <sub>2</sub> left in atmosphere, $\mu$ l	481	373	215	145	81	61	30
CO <sub>2</sub> dissolved, $\mu$ l	52	40	23	16	9	7	3
CO <sub>2</sub> fixed by cells, $\mu$ l	121	241	416	493	564	585	633
CO <sub>2</sub> fixed per 10 min over interval $\mu$ l	186*	160	88	38	12	3	0.5

\* The time during which CO<sub>2</sub> was taken up in the first interval was but 6.5 minutes instead of 10. The lost 3.5 minutes represents the period over which CO<sub>2</sub> was added to the flask.

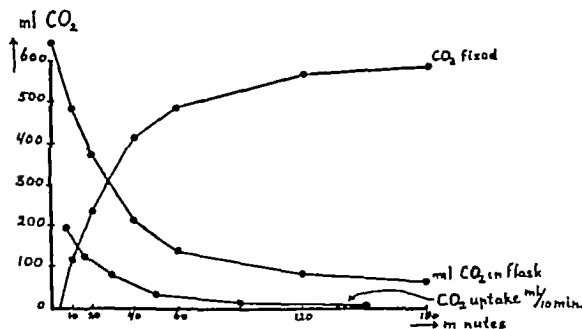


FIG 1 CO<sub>2</sub> fixation by resting cells of *Thiobacillus thiooxidans* in the absence of sulfur

In Fig 1, the fixation of CO<sub>2</sub> by the cells appears from the curve "CO<sub>2</sub> fixed". It begins at a rapid rate which gradually decreases. During the first hour 78 per cent of all the CO<sub>2</sub> which was to be taken up by the cells had been fixed. The character of the CO<sub>2</sub> fixation is better studied from the line representing the rate of CO<sub>2</sub> fixation (in milliliters per 10 minutes over the interval recorded). If this curve is compared with the curve representing the amounts of CO<sub>2</sub> present in the flask at these intervals (which is proportional to the



partial pressure of  $\text{CO}_2$ ), it is apparent that they are almost parallel. This could be taken as evidence that the rate of  $\text{CO}_2$  fixation is a function of the  $p\text{CO}_2$ . But if  $\text{CO}_2$  fixation is a function of  $p\text{CO}_2$ , the nature of the fixation reaction is more clearly defined. It could be explained either by assuming that the  $\text{CO}_2$  fixed is dissolved in the living cells (but not by the dead cells) in a manner expressed by the simple form of the Langmuir adsorption isotherm

$q = \frac{ap}{b + p}$  where  $q$  is the amount of gas dissolved under a partial pressure  $p$  and  $a$  and  $b$  are constants, or by assuming that the fixation of  $\text{CO}_2$  is a reversible enzymatic reaction. If  $\text{CO}_2$  fixation be considered readily reversible and dependent upon  $p\text{CO}_2$ , it probably requires little energy.

Several experiments yielding entirely similar results to the one reported have been done, particularly in the study of the question of how much  $\text{CO}_2$  could be taken up by cells under these conditions. This amount varies with the physiological condition of the culture but the maximum amount of  $\text{CO}_2$  that can be taken up by cells in the absence of sulfur was found to be about 40  $\mu\text{l}$  per 100 micrograms of bacterial nitrogen in suspensions which had recently been oxidizing sulfur. In the case of the experiment described in

table I, therefore, the maximum amount of  $\text{CO}_2$  that could have been synthesized would be 800  $\mu\text{l}$ , so that the 600–700  $\mu\text{l}$  added were less than the amount the cells could take up. This raises another problem. The suspension used in this experiment had been freshly harvested during which time it was exposed to  $\text{CO}_2$  in the atmosphere. It could, therefore, take up  $\text{CO}_2$  from its environment. On the other hand, the amount of  $\text{CO}_2$  that can be taken up by such suspensions is limited. Why, then, are these suspensions able to take up more  $\text{CO}_2$  when placed in Dixon-Keilin flasks?

It appears that the explanation may be found in the partial pressure of  $\text{CO}_2$  in the atmosphere. It will be noted that the amount of  $\text{CO}_2$  in the flasks at the end of their  $\text{CO}_2$  uptake period was slightly higher than that of the laboratory air. After the partial pressure of  $\text{CO}_2$  had been increased by the artificial addition of  $\text{CO}_2$ , this extra  $\text{CO}_2$  was taken up by the cells until they had reached approximately the same equilibrium with respect to  $p\text{CO}_2$  which had existed previously. The amounts of  $\text{CO}_2$  added were not sufficient to saturate the organisms, if they had been, only a definite and constant amount of  $\text{CO}_2$  would have been taken up. It therefore seems probable that within the limits represented by no  $\text{CO}_2$  and saturation, the  $\text{CO}_2$  is taken up by a reaction which reaches the equilibrium with the  $p\text{CO}_2$  of the environment in which the cells have been grown.

One further point of interest in the data of Table I, is the absence of appreciable  $\text{CO}_2$  liberation during the respiration in flasks 2 and 3. The amount of  $\text{CO}_2$  recovered from the atmosphere at the end of the respiration was very close to that originally present in the laboratory air, hence the oxygen uptakes

recorded are actually oxygen uptakes in spite of the fact that no KOH was present during this period

### *CO<sub>2</sub> Fixation under Anaerobic Conditions*

Since CO<sub>2</sub> fixation is not limited to the time during sulfur oxidation, but occurs in the absence of oxidizable sulfur, it was of interest to determine whether CO<sub>2</sub> fixation would occur in the absence of oxygen. Data upon this problem are recorded in Table III. Here the same suspension was employed as in the experiment recorded in Table I. In flasks 3 and 4 a suspension of sulfur was placed in the side arm. The flasks were flushed with hydrogen until no oxygen was available equilibrated, and the sulfur was added under hydrogen (so that no oxidation could occur). Known quantities of CO<sub>2</sub> were supplied, allowed

TABLE III  
*CO<sub>2</sub> Uptake in Absence of Oxygen*

200 microgram bacterial N per flask

Flasks	5 ml. suspension	CO <sub>2</sub> uptake		
		Under H <sub>2</sub>	O <sub>2</sub> uptake over 2 hrs	Under H <sub>2</sub>
		<i>μl.</i>	<i>μl</i>	<i>μl.</i>
1	Endogenous	75	4	0
2	Endogenous	71	4	1
3	+ sulfur	70	600	61
4	+ sulfur	74	600	56

The oxygen uptakes recorded are estimates based on values for endogenous respiration and S oxidation observed with the same suspension.

to act for 2 hours, and then reabsorbed. The CO<sub>2</sub> actually fixed in the cells is essentially the same in the presence or absence of sulfur and can occur in the absence of oxygen.

After the residual CO<sub>2</sub> was absorbed, the flasks were flushed with CO<sub>2</sub>-free air. After the oxygen uptake had continued for 2 hours the atmosphere was once more replaced with hydrogen and again the amount of CO<sub>2</sub> that could be fixed was determined. No attempt was made to measure the O<sub>2</sub> uptake during the 2 hour interval in which the gases were changed since the time necessary to change the atmosphere makes such measurements inaccurate. Instead, the values in Table III for oxygen uptake are based upon the values observed in other flasks of the same suspension, and hence only approximate the actual oxygen uptake during this interval. The actual oxygen uptake in the flasks was probably much less.

After the 2 hour respiration period, the flasks containing no sulfur did not regenerate the ability to take up CO<sub>2</sub> anaerobically, but the very small amount

of  $O_2$  uptake makes it impossible to be certain of this point. However, suspensions saturated with  $CO_2$  in other instances were found to have lost the faculty of fixing any more  $CO_2$  even after days of endogenous  $O_2$  uptake so that it seems probable that endogenous respiration cannot regenerate the ability to fix  $CO_2$ . Indeed, such suspensions exhibit  $CO_2$  liberation during the process of endogenous  $O_2$  uptake, indicating a true endogenous breakdown of organic compounds.

Sulfur oxidation in flasks 3 and 4, however, restored the ability of the suspension to fix  $CO_2$ , at least partially. It may be noted that under the experimental conditions probably not more than half the cells were actually attached to the sulfur particles. These experiments have been repeated several times. From them one can only conclude that *it is possible to oxidize sulfur in the absence of  $CO_2$  and to store up the energy within the cell where it can later be used for  $CO_2$  fixation under conditions during which sulfur oxidation is impossible*.

In the data contained here, hydrogen was used as an "inert" gas. Hydrogen was in no case taken up during  $CO_2$  fixation and thus could not have been active in any reaction. The  $CO_2$  uptake under hydrogen was found to be the same as under nitrogen. The reduction of  $CO_2$  by hydrogen which has been observed in other organisms thus does not seem to be important in *Thiobacillus thiooxidans*. Hydrogen was used more frequently than nitrogen in these experiments merely because we had a convenient source available, but the results were the same in pure nitrogen.

### *$CO_2$ As an Oxidizing Agent*

In a previous paper (Vogler *et al.*, 1942) evidence was given which indicates that the oxygen contained in the sulfate formed during sulfur oxidation might arise from the water in the medium rather than from the oxygen of the air. It therefore became of some interest to determine whether the  $CO_2$  might not also act as an oxidizing agent. The  $CO_2$  uptake during sulfur oxidation is a continuous process necessary for the supply of carbon to the cell. In this case the  $CO_2$  uptake must be accounted for, either in terms of an increased level of cell oxidation or in the excretion of oxidized products. If one assumes that the overall oxidation level of the cell is about that of carbohydrate (0) and that the  $CO_2$  is converted into cell material, it follows that for one molecule of  $CO_2$  there will become available one molecule of  $O_2$ . If, then, sulfur oxidation be measured in the presence of  $CO_2$ , and if a molecule of oxygen be "liberated" for each  $CO_2$  the  $Q_{O_2}(N)$  should decrease while the total uptake of  $O_2 + CO_2$  should be relatively constant.

Data which show this actually to be the case are given in Table IV. During oxidation of sulfur the  $Q_{O_2}(N)$  is decreased by the presence of  $CO_2$  (compare  $Q_{O_2}(N)$  of flasks 1 and 2 (no  $CO_2$ ) with flasks 3-8 (with  $CO_2$ )), yet the sum of  $Q_{O_2} + Q_{CO_2}$  is about constant (column 3). This indicates that for each mole-

cule of  $\text{CO}_2$  fixed, one less molecule of oxygen was taken up from the gas phase during sulfur oxidation

It could not be determined whether the  $\text{O}_2$  (which is thus apparently derived from  $\text{CO}_2$ ) was set free into the medium or whether it was used directly in sulfur oxidation without such release. Since, however, during endogenous respiration we can find no  $\text{O}_2$  liberated during  $\text{CO}_2$  fixation and since under hydrogen or nitrogen no  $\text{O}_2$  is formed in the presence or absence of sulfur, it may be assumed that the actual liberation of  $\text{O}_2$  into the medium or the gas phase does not occur

TABLE IV  
*CO<sub>2</sub> As Oxidizing Agent in Sulfur Oxidation*

Flasks	$\text{O}_{\text{O}_2}$	$\text{O}_{\text{CO}_2}$	$\text{O}_{\text{O}_2} + \text{O}_{\text{CO}_2}$	Time
				hrs
1	1580	0	1580	2
2	1600	0	1600	2
3	1020	710	1730	2
4	1490	283	1773	$\frac{1}{2}$
5	1270	320	1590	2
6	1140	540	1680	2
7	1220	420	1640	2
8	1100	570	1670	2

Flasks 1 and 2 had no  $\text{CO}_2$  added to the atmosphere. Flasks 1, 3, and 4 contained freshly harvested suspension. Flasks 2, 5, 6, 7, and 8 contained the same suspension aerated for 12 hours.

#### *CO<sub>2</sub> Uptake during Sulfur Oxidation*

The course of  $\text{CO}_2$  fixation during sulfur oxidation is exceedingly complex. The previous sections have shown that the  $\text{CO}_2$  may be fixed by the cells without sulfur, that, in the absence of oxygen, the sulfur does not aid  $\text{CO}_2$  uptake, and that the presence of  $\text{CO}_2$  may cause a lowering of the oxygen uptake. Yet the anaerobic experiments have shown that the energy of sulfur oxidation may be stored in the cell for a time, and used at a later time to fix  $\text{CO}_2$ . Thus the presence of  $\text{CO}_2$  in the atmosphere results in  $\text{CO}_2$  fixation which would deplete this stored energy supply. One might expect, therefore, that  $\text{CO}_2$  would accelerate the overall rate of sulfur oxidation.

These changes are not always apparent in a single determination since they tend to compensate for one another. That they do occur, however, is apparent from the data plotted in Fig. 2. The points on these curves were obtained with a 10 ml. fresh bacterial suspension containing 200 micrograms bacterial nitrogen. The same suspension was used in parallel flasks for the determination of the other points recorded. The total uptake observed during

sulfur oxidation (which is due to both  $\text{CO}_2$  and  $\text{O}_2$  uptake) is plotted in curve 1. The oxygen uptake in the absence of  $\text{CO}_2$  was obtained from a parallel series of flasks containing KOH (curve 2). The  $\text{CO}_2$  uptake at intervals in a parallel series of flasks (curve 3) was obtained by the methods described previously. It has been corrected for  $\text{CO}_2$  solubility and represents only  $\text{CO}_2$  fixation. The  $\text{CO}_2$  uptake by this suspension shows a rapid onset which, after about half an hour settles to an even rate of  $\text{CO}_2$  fixation. This is the result of two processes (1) the endogenous  $\text{CO}_2$  fixation (which has been drawn on the basis of the

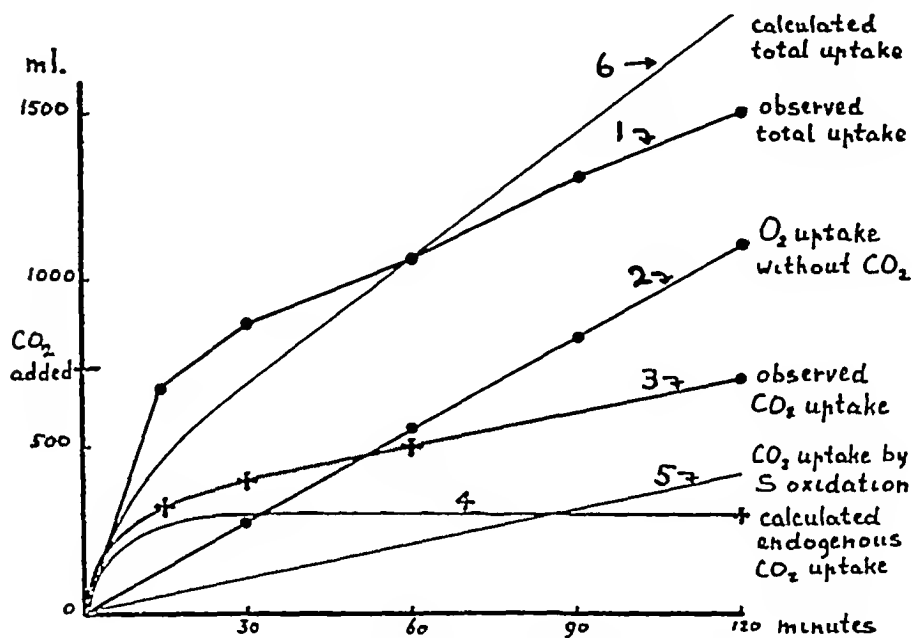


FIG 2 The course of  $\text{CO}_2$  fixation during sulfur oxidation by *Thiobacillus thiooxidans*. See text

value determined at 2 hours and the data of Fig 1 (shown in Fig 2 as curve 4)), and (2) the  $\text{CO}_2$  fixation due to sulfur oxidation (which has been drawn in as curve 5 by subtracting curve 4 from curve 3). From the observed  $\text{CO}_2$  uptake (curve 3) and the rate of  $\text{O}_2$  uptake in parallel flasks without  $\text{CO}_2$  (curve 2) the total uptake of  $\text{CO}_2$  and  $\text{O}_2$  which should be observed in the flask containing  $\text{CO}_2$  can be calculated (curve 6). Comparison of this curve (No 6) with the curve representing the actually observed uptake (No 1) shows that during the first hour the observed uptake exceeds the calculated total uptake, which indicates a marked stimulation of sulfur oxidation by the presence of  $\text{CO}_2$ . During the second hour the situation is reversed which indicates a depression in the oxygen taken out of the gas phase by the presence of  $\text{CO}_2$ . This probably means a utilization of the oxygen of  $\text{CO}_2$  for sulfur oxidation.

One may therefore conclude that in the early periods of sulfur oxidation the  $\text{CO}_2$  is not utilized as an oxidizing agent but stimulates the rate of sulfur oxidation. After saturation of the endogenous capacity of  $\text{CO}_2$  fixation, the oxidizing character of  $\text{CO}_2$ , resulting in a lower  $\text{O}_2$  uptake, obscures this stimulating effect.

### *Inhibition of $\text{CO}_2$ Fixation*

Four inhibitors were tested at concentrations which inhibit sulfur oxidation (Vogler *et al.*, 1942) to determine their effect on  $\text{CO}_2$  fixation.

The procedure consisted of a determination of the  $\text{Q}_{\text{O}_2}(\text{N})$  on sulfur of a dilute suspension containing 20 micrograms bacterial nitrogen per ml. This was determined over a period of 1 hour. The inhibitor was then added from the side arm to reach a final concentration indicated in Table V. The inhibited rate of sulfur oxidation was measured.  $\text{CO}_2$  was then added. The

TABLE V  
*Inhibition of  $\text{CO}_2$  Fixation*

Inhibitor	Inhibition	
	$\text{O}_2$ uptake	$\text{CO}_2$ uptake
	per cent	per cent
Na azide $\mu/100$	100	0
Na arsenite $\mu/100$	90	0
Na iodoacetate $\mu/10,000$	10	100
Na pyruvate $\mu/150$	100	100

extra uptake observed in the presence of the  $\text{CO}_2$ , less the oxygen taken up in parallel flasks containing no  $\text{CO}_2$  was taken as a rough measure of the  $\text{CO}_2$  fixed.

As appears from Table V, the inhibition of sulfur oxidation by pyruvate and sodium iodoacetate inhibits  $\text{CO}_2$  uptake completely. The inhibition by iodoacetate in this case is more extensive on  $\text{CO}_2$  fixation than on sulfur oxidation. Sodium azide and sodium arsenite which inhibit sulfur oxidation did not inhibit  $\text{CO}_2$  uptake. However, the  $\text{CO}_2$  uptake under these conditions must be considered as equivalent to the endogenous  $\text{CO}_2$  fixation. Inhibition of sulfur oxidation must in any case eventually inhibit  $\text{CO}_2$  fixation when the energy supplies of the cells have been depleted and the only energy for  $\text{CO}_2$  fixation is that coming directly from sulfur oxidation.

Effects similar to that of pyruvate seem to be exerted on sulfur oxidation by lactic, succinic, and fumaric acids. Citric acid occasionally has a similar but smaller effect. malic acid has little or no influence. The apparent influence of these organic acids on sulfur oxidation (which has not been found with any other organic substrate) serves as an indication that they are somehow involved

in the processes of sulfur oxidation and  $\text{CO}_2$  fixation. Further work is in progress on these materials. The indication is the more interesting since  $\text{CO}_2$  fixation in heterotrophic organisms is known to be related to the action of some of these same compounds.

#### DISCUSSION

The studies described in the previous pages have defined, to some extent, the character of the  $\text{CO}_2$  fixation process in the autotrophic bacterium. Of considerable interest is the demonstration that it is possible to oxidize the sulfur in the absence of  $\text{CO}_2$  and to store the energy thus obtained within the cell where it can later be used for  $\text{CO}_2$  fixation under conditions which do not permit sulfur oxidation. It is thus possible to separate the process of energy absorption (sulfur oxidation) from energy release ( $\text{CO}_2$  fixation) and to study each independently.

The chemosynthetic process has been thought to be related to the photosynthetic process. The data obtained with the autotroph should now enable one to make some estimate of the extent of this relationship. However, according to the concepts developed by students of photosynthesis, the energy obtained from light is directly transmitted from the activated chlorophyll to an intermediate in the conversion of  $\text{CO}_2$  to carbohydrate. These concepts leave no room for the storage of radiant energy within the cell in some form that can later be released for  $\text{CO}_2$  fixation. Upon this basis, therefore, the chemosynthetic and photosynthetic processes are distinctly different in that in the former such a storage of energy occurs while in the latter no such storage is possible. On the other hand, there seem to be no experiments which have directly tested this hypothesis in photosynthesis, apparently the very direct connection between light energy and  $\text{CO}_2$  fixation has almost always been gratuitously assumed. The available data on photosynthesis are not sufficient to exclude the possibility that the  $\text{CO}_2$  fixation process in the autotrophic bacteria is directly related. Therefore the data obtained with the autotrophic bacteria raise the definite question whose answer does not appear to be already available and whose study may open new paths towards an understanding of photosynthesis, namely, *is it possible to irradiate photosynthetic organisms in the absence of  $\text{CO}_2$  and to store at least a portion of the radiant energy within the cell in a form which can later be used for  $\text{CO}_2$  fixation in the dark?*

#### SUMMARY

In a study of chemosynthesis (the fixation of  $\text{CO}_2$  by autotrophic bacteria in the dark) in *Thiobacillus thiooxidans*, the data obtained support the following conclusions:

1.  $\text{CO}_2$  can be fixed by "resting cells" of *Thiobacillus thiooxidans*, the fixation is not "growth bound."

2. The physiological condition of the cell is of considerable importance in determining  $\text{CO}_2$  fixation.

3  $\text{CO}_2$  fixation can occur in the absence of oxidizable sulfur in "young" cells. The extent of this fixation appears to be dependent upon the  $p\text{CO}_2$ .

4  $\text{CO}_2$  fixation can also occur under anaerobic conditions and the presence of sulfur does not influence such fixation.

5 However, in the  $\text{CO}_2$  fixation by cells in the absence of sulfur, only a limited amount of  $\text{CO}_2$  can be fixed. This amount is approximately 40  $\mu\text{l}$ .  $\text{CO}_2$  per 100 micrograms bacterial nitrogen. After a culture has utilized this amount of  $\text{CO}_2$  it no longer has the ability to fix  $\text{CO}_2$  but releases it during its respiration.

6 Relatively short periods of sulfur oxidation can restore the ability of cells to fix  $\text{CO}_2$  under conditions where sulfur oxidation is prevented.

7 It is possible to oxidize sulfur in the absence of  $\text{CO}_2$  and to store the energy thus formed within the cell. It is then possible to use this energy at a later time for the fixation of  $\text{CO}_2$  in the entire absence of sulfur oxidation.

8 Cultures of *Thiobacillus thiooxidans* respiring on sulfur utilize  $\text{CO}_2$  in a reaction which proceeds to a zero concentration of  $\text{CO}_2$  in the atmosphere.

9  $\text{CO}_2$  may act as an oxidizing agent for sulfur.

10 Hydrogen is not utilized by the organism.

11 It is possible to selectively inhibit sulfur oxidation and  $\text{CO}_2$  fixation.

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# FURTHER CHEMICAL STUDIES ON BLOOD-AQUEOUS HUMOR DYNAMICS

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## INTRODUCTION

Through the use of heavy water as a tracer for ordinary water, and the radioactive isotopes of sodium ( $\text{Na}^{24}$ ), chloride ( $\text{Cl}^{36}$ ), and phosphorus ( $\text{P}^{32}$ ), the rate of accumulation of these substances in the anterior chamber of rabbit eyes after injection into the peritoneal cavity has been measured and reported previously (1, 2). In these earlier studies it was shown that water in the blood stream enters the anterior chamber at a rate equal to approximately 50 c. mm. per minute, whereas, the rate of accumulation<sup>1</sup> of sodium and chloride in the anterior chamber was such as to indicate an apparent rate of entry from the blood equivalent to 4.5 c. mm. per minute of whole aqueous humor. (The analogous rate for phosphorus was considerably less, but could not be determined exactly.) It appeared from these results that the rate of water movement into the anterior chamber is independent of the rate of movement of the electrolytes, i. e. the aqueous humor is not formed as a whole, but represents a summation of a series of separate equilibria.

The present studies have several objectives: first, to determine the rate of accumulation of various other substances in the anterior chamber from the blood, secondly, to establish where possible, the rate of transfer of the materials tested from the blood to the anterior chamber, and third, to determine which mathematical concept (ultrafiltration or secretion) of blood aqueous fluid dynamics the experimental findings fit best.

The first part of this report (the present paper) will be limited to the experimental work, while the second part (the following paper), will consist of an analysis of the experimental data based on a mathematical representation of several possible mechanisms of blood-aqueous dynamics.

The rate of accumulation following introduction into the blood, of the following substances has been investigated:  $\text{SCN}$ ,  $\text{Br}$ ,  $\text{Li}$ ,  $\text{PO}_4$ , urea, levulose,  $\text{Mg}$ , and  $\text{Fe}$ .

Asher (3) found an increase in the total halogen content of the anterior chamber of a patient treated with bromides and inferred that the blood-aqueous

<sup>1</sup> The use of this term is made necessary from the fact that the rate of transfer of a material into the anterior chamber may or may not correspond to its rate of accumulation. It was only the latter which was measured in the sodium and chloride studies.

barrier was permeable to bromides Lipschitz (4) found an equilibrium ratio of aqueous humor to blood bromide of about 1.08 when sodium bromide was administered orally to rabbits

Walker (5) records that the inorganic phosphate content of the aqueous averaged 37 per cent of that of the serum He used fowls, rabbits, dogs, and cats Tron (6) found the inorganic phosphate of ox eyes to be 60 per cent of the plasma Duke-Elder (7) reported that the aqueous contained 110 per cent of the phosphate found in the blood serum of horses

The ratio of urea in the aqueous to that in the blood has been reported to be about 1.0 by Duke-Elder (7), 0.68 by Walker (5), 0.75 by Adler (8), and 0.90 by Benham (9) Moore *et al* (10), using cats, injected urea into the blood and found that the concentration in the anterior chamber increased slowly, requiring about 6 to 9 hours before the concentration of urea in the aqueous reached that of the plasma Robertson (11, 12) has injected 25 per cent urea intravenously into cats in combination with 50 per cent glucose and 5 per cent creatinine He found that, starting from an initial ratio of urea in the anterior chamber to that in the blood of 76 per cent (2 animals), the new final equilibrium ratio was approximately 35 per cent as seen from his chart or slightly more, approximately 45 per cent as calculated from his table which shows a typical experiment

The ratio of Mg in the anterior chamber, compared with the blood was found to be 0.64 by Tron (6), 0.91 by Duke-Elder (7), and 0.95 by Sary and Winternitz (13) Incidentally, the latter authors observed that only 82 per cent of the serum Mg was ultrafilterable

### Methods

Unanesthetized rabbits (large majority 2 to 3 kg albinos) were used for all of the experiments Most of the test substances were administered by one intraperitoneal injection Exceptions to this were levulose and urea in which instances it was desired to keep the concentration in the blood as nearly constant as possible For these experiments an intravenous injection was followed immediately by one given intraperitoneally The latter procedure had to be repeated for the levulose experiments at about 20 minute intervals, the dose being adjusted to keep the level in the blood approximately constant

The substances used, the approximate amounts, and the method of introduction employed for all of the test agents are tabulated on page 121

All blood samples were withdrawn by cardiac puncture and all aqueous samples were removed under local anesthesia (2% pontocaine) by means of calibrated microsyringes Primary aqueous only was used in all of the experiments

In order to determine whether some of the test materials are present in plasma in a non-diffusible state *i.e.* bound, most of these materials were ultrafiltered through cellophane Plasma containing a known concentration of the substance was placed in an apparatus consisting of two small chambers separated by a cellophane membrane which was supported by a perforated brass screen Air pressures up to 100

Substances	Route	Amount injected per kg body weight	Concentration of injected solution
		ml	per cent
SCN as NaSCN	Intraperitoneally	10	$\frac{1}{2}$
Br as NaBr	Intraperitoneally	15	3
Li as LiCl	Intraperitoneally	20	3
PO <sub>4</sub> as phosphate buffer pH 7.0	Intraperitoneally	10	3
Urea	Intravenously	1	20
	Intraperitoneally	2.5	8
Levulose	Intravenously	1	10
	Intraperitoneally	20	5 (repeated in some)
Mg as MgCl <sub>2</sub>	Intraperitoneally	3	10
Fe as FeSO <sub>4</sub>	Intraperitoneally	10	2
and Ferric NH <sub>4</sub> citrate	Intraperitoneally	20	2

lbs./sq inch were applied and the ultrafiltrate was collected in such manner that there was no opportunity for evaporation. After 15 to 20 per cent of the total volume had passed through the filter the filtrate and remaining plasma were analyzed. After adjusting for the relative water contents the fraction which was ultrafilterable was calculated from the starting concentration in the plasma and the concentration in the filtrate. The method of calculation is illustrated in the following example. SCN was added to plasma to a concentration of 96 micrograms per gm of water and after 20 per cent of the volume had ultrafiltered, the plasma contained 103 micrograms per gram of water and the ultrafiltrate 74 micrograms per gm. of water. Thus 77 per cent was considered to be ultrafilterable. (The concentration found in filtrates collected at various times throughout the ultrafiltration procedure did not vary appreciably.)

The question of bound test substances in plasma was also examined by a differential dialysis method which was designed to avoid errors arising in the usual dialysis methods from considerable dilution of the plasma and excessive changes in the concentration of the substance being tested. The method consists of dialyzing plasma containing a known concentration of the test material against the same concentration of the test material in isotonic saline. Under these conditions the freely diffusible portions of the test agent would be expected to approach equality on either side of the membrane irrespective of any part which may be bound in the plasma. Thus when equilibrium is reached the bound portion which must remain on the plasma side of the membrane will be apparent as a difference in the total concentration of the test substance on the two sides. In the case of the anions, the Gibbs-Donnan effect would tend to produce a small difference in concentration in the opposite direction i.e. higher on the saline side. Since we are here interested in quantities considerably greater than any which would result from the Donnan effect no attempt was made to take into account the influence of the Donnan effect on the concentrations.

An outline of the analytical procedures used in these experiments follows. The errors of the methods are expressed as the average deviation from theoretical values at

concentration levels such as were found in the plasma and aqueous of the experimental animals. This deviation is expressed in per cent.

**SCN**—Plasma and aqueous humor thiocyanate concentrations were determined by the well known ferric nitrate colorimetric method. The plasma analyses were performed photoelectrically using a Jena BG-7 (2 mm) filter. Standards were prepared by adding known amounts of NaSCN to water and to normal plasma. When corrected for the plasma blank identical curves resulted which deviated only slightly from Beer's law. Average agreement between duplicates in a series of 28 determinations was 1.5 per cent with the greatest deviation 3.4 per cent. Thiocyanate in deproteinized aqueous samples was determined by the same method, except that the quantities available necessitated visual color comparison. In order to compensate automatically for the aqueous blank (about 7 micrograms per gm. of water) standards were made up with corresponding quantities of normal aqueous present. Accuracy of analysis of aqueous SCN was approximately  $\pm 5$  per cent.

**Br**—The concentration of bromide in plasma and aqueous humor was determined by a photoelectrometric adaptation of the gold chloride method of Wuth (14), thereby decreasing the error from more than 15 per cent to less than 5 per cent.

Plasma proteins were precipitated with trichloroacetic acid and the bromide content of the filtrate was measured with a photoelectric colorimeter using a Jena BG-7 (2 mm) filter. Series of standards, made by adding known amounts of sodium bromide to water and to plasma up to 1400 micrograms per gm. of water gave identical curves when corrected for the plasma blank and deviated only slightly from Beer's law when the above filter was used. Bromide concentration in the aqueous was determined by visual matching, because the volumes were too small for use in the colorimeter. The color produced by gold chloride after removal of proteins with trichloroacetic acid was compared with that of a series of standards made by adding known amounts of sodium bromide to normal blank aqueous and treating in the same way as the unknowns. Standards were made with aqueous humor present to compensate for the slight fading of gold chloride caused by some constituent of aqueous, probably ascorbic acid. With this procedure the accuracy of aqueous humor analyses was  $\pm 6$  per cent.

**Li**—Plasma and aqueous humor lithium contents were determined for us by Prof. G. R. Harrison of the Massachusetts Institute of Technology by quantitative spectrophotographic analysis. The average amount of lithium found to be present in pooled normal aqueous humor and plasma was 4 micrograms per gm. of water.

**PO<sub>4</sub>**—The inorganic phosphate of both plasma and aqueous humor was measured in trichloroacetic acid filtrates by the method of Tschopp and Tschopp (15). Using a photoelectric colorimeter equipped with a Wratten 61N filter, the light transmission was found to bear a constant logarithmic relationship to the concentration of phosphate in accordance with Beer's law.

The average agreement in the PO<sub>4</sub> content of six paired aqueous samples was  $\pm 2$  per cent with the greatest difference of 6.3 per cent.

**Urea**—Urea was determined by the aeration-titration method following digestion with urease. With one exception, the average deviation in a series of thirteen control analyses at several different concentrations was  $\pm 2$  per cent. Aqueous removed

from both eyes as nearly simultaneously as possible was pooled in order to increase the accuracy of these results

*Levulose*—Levulose was determined by a modification of the method of Corcoran and Page (16). Plasma proteins were precipitated without heating by addition of 0.5 ml. of 4.5 per cent  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  to 0.5 ml. plasma plus 2.5 ml. water. To this was then added 0.5 ml. of 0.2 *N* NaOH. 1 ml. of filtrate was heated in a boiling water bath with 5 ml. of diphenylamine-acid-alcohol reagent for 30 minutes in graduated tubes. Only the lower inch of the tubes was immersed so that the upper portion of the tubes remained relatively cool; this technique caused the alcohol vapors to condense and reflux and avoided errors arising from precipitation of the reagent. After cooling the volume was made up to 10 ml. with ethyl alcohol and the color was measured photoelectrically using a Wratten 61N filter.

Analyses of aqueous were done in the same way except that the protein precipitation stage was omitted; the aqueous sample simply being made up to 1 ml. with water and heated with the diphenylamine reagent. The small blank due chiefly to some reaction of the reagent with glucose in plasma and aqueous was determined for each animal and subtracted from the experimental values. Recoveries of levulose from plasma were 97 to 105 per cent and 96 to 108 per cent from aqueous. Plasma duplicates agreed to within  $\pm 2$  per cent.

*Mg*—Determinations of magnesium by the Titan yellow method as described by Gillam (17) were carried out on tungstic acid plasma filtrates and whole aqueous. The samples were diluted in order to bring them within the useful range of the test (0 to 4 micrograms per ml.) and the colors were matched visually with a series of standards.

Recovery of magnesium from plasma in the concentrations met with in the experiments showed an average agreement of  $\pm 5.2$  per cent in seven cases, and a maximum error of 20 per cent (one case). In duplicate plasmas the average variation was 4.1 per cent while in duplicate control aqueous it averaged 7 per cent.

*Fe*—Iron in plasma was determined by using sulfosalicylic acid to precipitate the proteins and produce a characteristic amethyst color. After centrifuging the color in the supernatant was determined photoelectrically. A Jena VG 2 mm. (green) filter was used. This method determines iron in the ferric state. By adding concentrated  $\text{NH}_4\text{OH}$  to the above supernatant in an amount sufficient to turn Congo red paper red both ferric and ferrous iron may be determined colorimetrically using a Wratten 47-C5 filter. Advantage was taken of this procedure to estimate the ferrous iron by difference. The accuracy is  $\pm 5$  per cent.

All concentrations in the aqueous and plasma have been expressed on the basis of a gram of water. In depicting the relative concentration of the test materials in the plasma and in the anterior chamber the maximum concentration in the plasma of each animal of the freely diffusible portion of the test agent has been arbitrarily called 100 per cent and all other concentrations have been adjusted proportionately. In the case of urea the plasma concentration was maintained fairly constant; the average concentration in place of the maximum concentration was called 100 per cent (Fig. 5). In this way the points scatter above and below the line representing 100 per cent. Thus each pair of curves shown in Figs. 1 to 5 is a composite of all the aqueous sam-

ples and all of the blood samples. Two aqueous samples and three or four blood samples were taken from each animal. In the case of levulose the variations in concentration which were encountered necessitated expressing the findings of these experiments in actual concentration instead of reducing the results to a percentage basis (Fig. 6)

### RESULTS

*SCN*—From four ultrafiltration and three differential dialysis experiments on plasma containing 100 micrograms of *SCN* per ml it was found that but 77 per cent of the *SCN* was present in a freely diffusible state. The individual experiments were ultrafiltration, 80, 79, 74, 74 per cent, dialysis, 77, 73, and 82 per cent

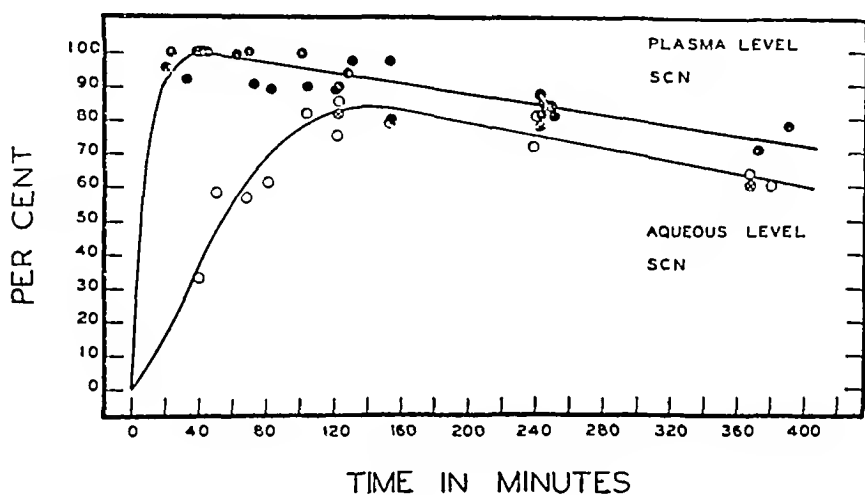


FIG 1

After correcting for the proportion of *SCN* in the plasma which is bound, the relative concentrations of freely diffusible *SCN* present in plasma and aqueous at various times after injection are shown in Fig. 1. The small initial concentration of *SCN* present in both plasma and aqueous has been subtracted from all of the concentrations in order to facilitate analysis in the following paper. The absolute maximum levels of *SCN* reached in the plasma varied between 85 and 135 micrograms per ml. From the figure it is evident that the *SCN* concentration of the aqueous rapidly approaches that of the freely diffusible form in the plasma. Whether it actually reaches that of the plasma in all instances is perhaps questionable, that it does in some appears quite probable. The circles enclosing an  $\times$  represent glaucomatous eyes. The significance of these points will be discussed in a following paper.

*Br*—Ultrafiltration and differential dialysis of plasma containing bromide in a concentration of 1200 micrograms per ml showed that on an average

92 per cent was freely diffusible. The separate analyses were ultrafiltration, 96, 87, and 90 per cent, dialysis, 91.5 and 92 per cent.

Fig 2 illustrates the relative concentration of freely diffusible bromide in the plasma and aqueous at various intervals after injection. The maximum level of bromide in the plasma varied from 1185 to 1450 micrograms per ml.

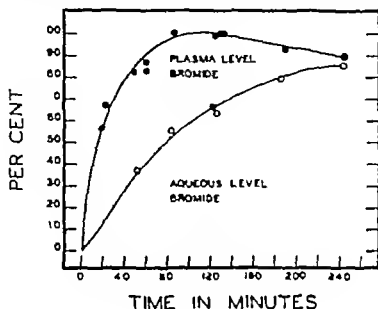


FIG. 2

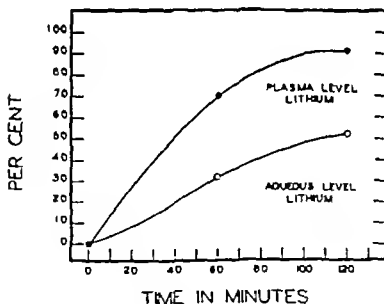


FIG. 3

*Li*—The expense of spectrographic analyses limited the number of experiments which could be performed using lithium as a test substance. In view of possible implications inherent in the use of a test agent having as small an atomic weight as Li, it is thought of some value, to report the results of the three plasma and three aqueous determinations. The relative concentration of lithium in the aqueous and plasma, following injection of LiCl, is shown in Fig 3. The absolute concentration as represented by the point in the plasma



curve at 90 minutes was 100 micrograms per gm of water. Normally present in the aqueous and plasma were 4 micrograms per gm of water. This amount was subtracted from the experimental results of both plasma and aqueous.

While a more quantitative comparison of the rate of transfer of  $\text{Li}$  from the blood to the anterior chamber will be presented in the following paper, it is clear that the rate of accumulation of  $\text{Li}$  is not conspicuously greater than was found for  $\text{Na}$ ,  $\text{Cl}$ ,  $\text{SCN}$ , or  $\text{Br}$ . The possibility that lithium is sufficiently hydrated to invalidate any conclusions regarding rate of penetration and molecular size has not been ruled out.

$\text{PO}_4$ —Phosphate, as phosphate buffer, pH 7.0, added to plasma was dialyzed by the method described above and found to be completely diffusible.

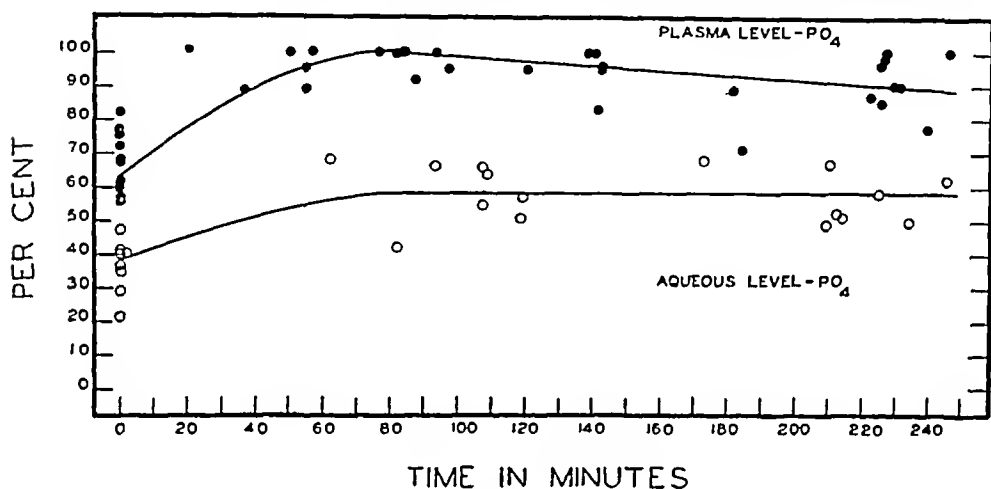


FIG 4

This is in agreement with the results of Walker (5) who used an ultrafiltration method.

Initially the inorganic phosphorus level was found to vary between 49 and 80.5 micrograms per gm of water in seventeen control plasmas, and between 33.6 and 45.2 micrograms per gm of water in thirteen control aqueous samples. The relative concentration of inorganic phosphorus in the plasma and aqueous of injected animals appears in Fig 4. These results, like those in which radioactive phosphorus was used (2) showed considerable fluctuations in concentration both in the aqueous and plasma. While exact quantitative treatment would be unwarranted it would appear from the figure that the initial equilibrium ratio ( $63 \pm 10$  per cent) of aqueous to plasma concentration was approximately maintained following the introduction of additional phosphate into the animal.

*Urea*—Urea was shown by differential dialysis to be 100 per cent diffusible. This result too, is confirmatory of that found by Walker and others who have employed ultrafiltration.

Initially the plasma urea concentration was found to vary between 340 to 470 micrograms per gm of water (ten cases), except in one instance where 590 micrograms per gm. of water were present. The aqueous to plasma concentration ratio was  $66 \pm 5$  per cent in four animals.

The findings resulting from combined intravenous and intraperitoneal injections of urea are shown in Fig 5. The starting concentration of urea is seen to correspond to 33 per cent when the average plasma concentration after injection is assigned a value of 100 per cent. From the figure it may be seen that the plasma concentration of urea remains practically constant throughout the test period and that the aqueous concentration increases at first, but like

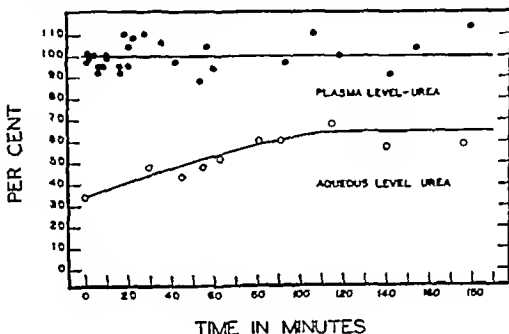


FIG. 5

$\text{PO}_4$ , appears to reestablish an equilibrium having the same ratio as was found initially *viz*, at approximately 65 per cent.

**Levulose**—Levulose was found to be in a freely diffusible state in plasma as determined both by ultrafiltration and differential dialysis.

From preliminary experiments it was found that the concentration of levulose in the plasma decreased rapidly following intravenous injection. Following intraperitoneal injection it rose and then fell rapidly. Even when administered repeatedly it was not found possible to maintain the concentration at a constant level nor at levels sufficiently alike in different rabbits to consider them all together. For these reasons the actual concentrations of levulose in plasma are shown separately in Fig 6. It may be seen from the curve showing the concentration in the aqueous that the final equilibrium ratio does not approach 100 per cent, but appears to be somewhat less than 50 per cent.

It is not possible to state the exact initial ratio of levulose in the anterior chamber to that in the plasma because the test used is not sufficiently specific for this sugar.

It is interesting to note that Robertson (11, 12) who measured the rate of increase in concentration of glucose after intravenous injection found that there was a "deficiency of at least 40 per cent of the blood sugar level in the aqueous " Weld in a similar study (18) found approximately the same deficiency for glucose and progressively greater deficiencies for sucrose and raffinose

Attempts were made to test the rate of accumulation of Mg in the anterior chamber after intraperitoneal injections of  $MgCl_2$  From 22 to 38 micrograms of Mg per gm of water (eleven cases) were present normally in the aqueous Unfortunately, the changes in concentration of this ion in the anterior chamber were not of sufficient magnitude, considering the accuracy of the analytical method, to be more than suggestive of the rates involved The inconstancy of the plasma levels also contributed to the difficulty of any interpretation

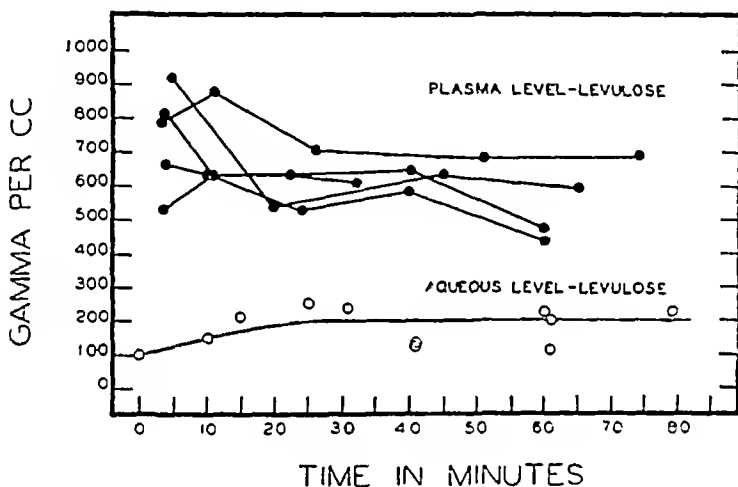


FIG 6

Dialysis of plasma to which had been added both ferrous and ferric iron in concentrations up to 700 micrograms per ml showed that practically all of the iron was bound The cellophane membranes used for the dialysis experiments were shown to be freely permeable to both forms of iron in separate experiments in which the plasma was omitted

The results of the animal experiments (in so far as they may be trusted, since the animals died after some hours) are consistent with this finding, for despite plasma iron concentrations as high as 700 micrograms per ml, the concentration of iron in the anterior chamber remained less than 0.5 microgram per ml for periods up to 5 hours

#### DISCUSSION

No attempt has been made to compare the rates of transfer of the substances tested into the anterior chamber, because of the obvious difficulty of mentally compensating for varying plasma concentrations The following paper will be concerned with this problem

## SUMMARY

The proportion of SCN, Br, PO<sub>4</sub>, urea, levulose, and Fe which remains freely diffusible when added to plasma has been determined by ultrafiltration and "differential" dialysis through a cellophane membrane. After injecting each of these substances as well as Mg and Li into rabbits a continuous record of their concentration in the plasma was obtained for each animal and the concentration in the aqueous humor was also determined and related to the maximum diffusible concentration in the plasma.

The authors wish to acknowledge the technical assistance of Miss Cynthia Steitz in carrying out these studies.

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# THE MECHANISM OF AQUEOUS HUMOR FORMATION INFERRED FROM CHEMICAL STUDIES ON BLOOD AQUEOUS HUMOR DYNAMICS

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Perhaps no problem in ocular physiology has received so much attention or been discussed so vehemently as the question of whether the aqueous humor is formed by ultrafiltration (dialyzation) or by some secretory process. One of the chief arguments cited for or against the ultrafiltration as opposed to the secretion theory has been based upon the equilibria which the constituents of the aqueous humor form with the blood. Strong support has been gained for the ultrafiltration hypothesis from the fact that with several notable exceptions ( $K$ ,  $PO_4$ ,  $SO_4$ ) the electrolyte distribution does not vary appreciably from that required by the Gibbs-Donnan equation for static equilibria.

On the other hand, it has always been assumed that the ultrafiltration hypothesis was incompatible with equilibrium ratios of freely diffusible substances which are appreciably less than 1, unless some special assumptions, such as utilization within the anterior chamber or selective reabsorption were invoked to account for the deficiency. Examples of compounds which appear to be present in lower concentrations in the aqueous humor than in the blood are phosphate and sulfate and such freely diffusible non-electrolytes as urea, uric acid, creatinine, sucrose, raffinose, and probably glucose. With the possible exception of the last named, there is no evidence that appreciable utilization of any of these compounds occurs within the anterior chamber nor that they are selectively reabsorbed.

For reasons which will be given subsequently the authors do not believe that it is permissible to draw any conclusions concerning the mechanism of aqueous formation merely from the aqueous humor blood equilibria ratios. Neither would it appear possible to estimate directly the relative rates of transfer of different substances from the blood to the anterior chamber from such ratios alone. Some of the additional data deemed necessary for the solution of these problems will be presented later.

The purpose of this paper is twofold: first, to point out mathematically the quantitative implications of the ultrafiltration and secretory hypotheses, and secondly, to analyze the experimental data already presented (1, 2) on the basis of such theoretical formulations, with the object of determining (a) the relative rates of transfer of substances into the anterior chamber, and (b) which hypothesis, if either, is supported by these data.

If aqueous humor is assumed to form either as a result of ultrafiltration or of some secretory phenomenon, there appear, in general, to be three separate processes<sup>1</sup> which could account for the equilibria between aqueous humor and blood. They are

1 Aqueous constituents separately may enter the anterior chamber from the blood by ultrafiltration where, under static conditions<sup>2</sup> they would accumulate until the concentration in the aqueous reaches that in the blood. In this instance the process would presumably obey Fick's law, *i.e.* the rate of transfer would be proportional to the difference in concentration on the two sides of the barrier or barriers, and would eventually lead to an equilibrium ratio of 1.0 (It is this process only, which is assumed, presumably, by those who use the ratios below 1.0 as evidence against the ultrafiltration theory.)

2 Aqueous constituents may enter the anterior chamber by ultrafiltration and escape by another mechanism, *e.g.*, flow.<sup>3</sup> It will be shown later that such a mechanism could account for any equilibrium ratio below 1.0.

3 Aqueous constituents may enter the anterior chamber from the blood by secretion, in this instance it is obviously necessary that there be some leakage out of the anterior chamber. Such leakage would presumably occur by a different route, and might be either a flow or conceivably another secretory process. In either instance the simplest assumption concerning the rate of leakage of any substance out of the anterior chamber is that it is proportional to its concentration in the aqueous humor. It will be shown that this process also could account for equilibrium ratios less than 1.0 and in addition could lead to equilibrium ratios above 1.0.

In summary, an aqueous constituent may form a static equilibrium with the blood by a process of ultrafiltration, or it may enter by ultrafiltration or secretion and leave the anterior chamber by means of a flow. Moreover, final equilibrium ratios below 1.0 could be accounted for by either the ultrafiltration or secretory hypothesis under the conditions set forth under paragraphs 2 and 3, *i.e.* some net flow (not exchange) out of the anterior chamber, furthermore

<sup>1</sup> It is, of course, possible that some combination of these processes, or others, may be the actual one involved in aqueous formation, but the point which we desire to emphasize especially is not how the aqueous is necessarily formed, but some of the additional factors which must be considered before inferring from analytical procedures what is the mechanism for aqueous formation.

<sup>2</sup> Static conditions are here assumed to be those in which there is no flow, or so called through and through circulation resulting in transfer of aqueous humor to outside the anterior chamber. The only loss of material from the aqueous under static conditions would occur by means of exchange such as occurs in dialysis.

<sup>3</sup> For the present purpose it does not matter whether the aqueous constituents are considered to enter through the ciliary body, the iris or elsewhere, nor does it matter whether they leave as a flow through Schlemm's canal or elsewhere so long as the site of entrance and exit are different.

such ratios are not readily explicable by either hypothesis under static conditions

While many investigators have recognized the possibility of flow out of the anterior chamber, and have designed experiments to measure its rate, no one to our knowledge has pointed out the effect such a flow would have on the equilibrium ratio for any particular aqueous constituent, other than to call attention to the fact that if there is a flow the Gibbs-Donnan equilibrium would not prevail (Robertson, Ridley, etc.)

Let us consider now the situation as outlined in paragraph 2 above, *viz*, materials enter the anterior chamber from the blood by filtration and in addition leave by a flow. Under these conditions substances would enter the aqueous at a rate proportional to the difference in concentration in the blood and anterior chamber, whereas, if they leave the anterior chamber by a flow process they would do so at a rate proportional to the actual concentration in the anterior chamber. In this simple case it is assumed that the blood level is constant, that the diffusion rates are rapid compared with the rates of transfer, and that the ratio of the area of the blood aqueous barrier to the volume of the anterior chamber remains constant for different eyes. Moreover, it is assumed that the relative ease with which water may move across the barrier in either direction, as shown in (3), permits the volume, hence the intraocular pressure, to remain essentially constant despite loss of fluid by means of flow.

The relation of the concentrations reached in the anterior chamber to the inflow and outflow may be seen from the equations which follow. Where

$A$  = amount of constituent present in aqueous humor,

$C_1$  = concentration in blood

$C_2$  = concentration in aqueous at a given time  $t$ ,

$k$  = coefficient of transfer out of anterior chamber by flow,

$k_1$  = coefficient of transfer from blood to anterior chamber

$t$  = time

$V$  = volume of the anterior chamber

The rate of change of amount of a given constituent in the anterior chamber =

$$\frac{dA}{dt} = k_1(C_1 - C_2) - k C_2 \quad (1)$$

$$C_2 = A/V \text{ whence}$$

$$\frac{d(VC_2)}{dt} = V \frac{dC_2}{dt} = k_1 C_1 - (k_1 + k) C_2$$

Integrating between the limits  $t = 0$  to  $t = t$  and rearranging, we have

$$C_2 = \frac{k_1}{k_1 + k} C_1 \left[ 1 - e^{-\left(\frac{k_1 + k}{V}\right)t} \right] \quad (2)$$



Equilibrium will occur when  $t = \infty$

$$\frac{C_2}{C_1} = \frac{k_2}{k_2 + k'} \quad (3)$$

From Equation 3 it is apparent that equilibrium ratios less than 1 may result for any freely diffusible substance without making any assumption other than that there is some flow out of the anterior chamber, *viz*,  $k' \neq 0$ . As suggested above, it follows that unless definite proof is forthcoming to show that there is no flow out of the anterior chamber, it appears that the ultrafiltration hypothesis can account for equilibrium ratios less than 1 and therefore need not be discarded because of experimental findings of such ratios.

Similarly, measurements of the equilibrium ratio do not give any indication of the rate of transfer of a substance from the blood into the anterior chamber, they give only the overall effect of net transfer by ultrafiltration into the anterior chamber and leakage out by flow.

In a similar manner let us now consider the situation when substances are presumed to enter the anterior chamber from the blood by a secretory process and leak out of the anterior chamber by a process of flow as described above under paragraph 3. It is again assumed, as the simplest case, that the concentration of the material in the blood remains at a constant level, and that the rate of secretion is proportional to the concentration present in the blood. All symbols have the same significance as before. The rate of change of amount of a given constituent in the anterior chamber equals

$$\frac{dA}{dt} = k_2 C_1 - k' C_2 \quad (4)$$

$$\frac{d(VC_2)}{dt} = V \frac{dC_2}{dt} = k_2 C_1 - k' C_2$$

Integrating between the limits from  $t = 0$  to  $t = t_1$ , and rearranging we have

$$C_2 = \frac{k_2}{k'} C_1 \left( 1 - e^{-\frac{k'}{V} t_1} \right) \quad (5)$$

and at  $t = \infty$

$$\frac{C_2}{C_1} = \frac{k_2}{k'} \quad (6)$$

In this instance too, the effect of a flow out of the aqueous is to complicate the situation, thereby making it impossible from the equilibrium ratio to determine either the rate of transfer from the blood to the anterior chamber, or to infer whether the transfer occurs as a result of filtration or secretion.

It should be evident that, since the blood-aqueous barrier is more permeable to water than to solutes constant volume conditions will be maintained and the effect of flow out of the anterior chamber is to reduce the quantity of solutes

below that which would be present were there no flow. This reduction in the quantity of solutes present will have no effect on the rate of secretion, which, by hypothesis is dependent solely on the concentration in the blood, but will have an effect on the rate of filtration which is dependent upon the difference in concentration between the aqueous humor and the blood and *a fortiori* on the concentration in the aqueous, since the concentration in the blood is assumed to be constant.

The question now arises whether it is possible from any experiments involving analyses of blood and aqueous humor to determine either the transfer rate from the blood to the aqueous, or to infer by what process the transfer is taking place. It seems that experiments involving analyses of blood and aqueous humor can permit such inferences to be drawn provided that the concentration of a given substance in the anterior chamber and in the blood is known at different periods of time following its introduction into the blood.

It is apparent that when  $k'$  (coefficient of out flow) is small relatively high concentrations in the anterior chamber could be obtained from relatively small values of  $k_2$  (coefficient of transfer in) while if  $k'$  is large, relatively high values of  $k_2$  would have to be assumed to account for the same experimental data.

If, for a given ratio of concentration in the aqueous to that in the blood, a value of  $k'$  is assumed, the corresponding value of  $k_2$  may then be calculated. Moreover, on assuming a different value of  $k'$ , other values of  $k_2$  will be found. Hence it is possible for any given ratio of concentrations to plot  $k_2$  against  $k'$ . At another time, when the ratio of the concentration in the aqueous to that in the blood is different, a new plot will be found, the slope of which will differ from the first one. If, therefore, we are to arrive at values for  $k'$  and  $k_2$  which are to be reasonably satisfactory for all of the experimentally observed concentration ratios, the specific values for  $k'$  and  $k_2$  must be selected which are found at the intersection of all of the plots.

The procedure just outlined was applied to concentration ratios in which the concentration of the substance under consideration was assumed to be at a constant level in the blood. From an experimental standpoint this condition is difficult to obtain, but if continuous records are made of the concentration in the blood, it is still possible although quite laborious to apply the same method of treatment to the resultant data, provided an equation can be developed which will describe the experimental data representing the concentration in the blood. Since the blood concentration varied for different test materials, as described in the preceding papers, it was necessary to derive several different equations in order to treat the data in the manner outlined above. These equations along with the methods used in applying them to the solution of the general problem will be described forthwith.

The concentration in the blood of radioactive isotopes of sodium and chloride (see (1)) and lithium (preceding paper) was found to increase to a maximum

and remain essentially stationary throughout the observation period. A good fit for the data may be had from the following equation

$$C_1 = C(1 - e^{-k_1 t_1}) \quad (7)$$

where  $C$  is the asymptotic blood constant and represents the maximum concentration level approached by the blood.  $K_1$  is the coefficient of transfer from peritoneal cavity to blood, the other symbols have the same meaning as before.

By substituting for  $C_1$  in equation 1 which describes the rate of change of concentration in the anterior chamber when the transfer is assumed to take place by filtration and takes into account a flow out of the anterior chamber, we have

$$\frac{dA}{dt} = k_2 [C(1 - e^{-k_1 t_1}) - C_2] - k' C_2 \quad \text{or}$$

$$\frac{dC_2}{dt} = \frac{k_2}{V} [C(1 - e^{-k_1 t_1}) - C_2] - k' C_2$$

Integrating from  $t = 0$  to  $t = t_1$

$$C_2 = k_2 C \left[ \frac{1}{k_2 + k'} \left( 1 - e^{-\frac{k_2 + k'}{V} t_1} \right) - \frac{1}{k_1 V - k_2 - k'} \left( e^{-\frac{k_2 + k'}{V} t_1} - e^{-k_1 t_1} \right) \right] \quad (8)$$

Again when  $t = \infty$

$$\frac{C_2}{C} = \frac{k_2}{k_2 + k'}$$

In a similar way the equation may be derived for treating the data on the basis of secretion—the condition outlined under paragraph 3 above. The resulting equation is

$$C_2 = k_2 C \left\{ \frac{1}{k'} \left( 1 - e^{-\frac{k_1 t_1}{V}} \right) - \frac{1}{k_1 V - k'} \left( e^{-\frac{k_1 t_1}{V}} - e^{-k_1 t_1} \right) \right\} \quad (10)$$

If, as was the case for the SCN and Br experiments, the level of the test substance in the blood reaches a maximum and then falls, the solution of the problem becomes more laborious, but still may be handled quantitatively with the aid of the following equation which empirically describes the concentration in the blood within the time limits investigated

$$C_1 = C(1 - e^{-k_1 t_1}) - k_3 t_1 \quad (11)$$

where  $k_3$  represents the coefficient of loss from blood. By substituting again for  $C_1$  in both Equation 1 (ultrafiltration) and Equation 4 (secretion) and integrating, we arrive at the following relation for ultrafiltration

$$C_2 = \left( \frac{k_2 C}{k_2 + k'} + \frac{k_2 k_3 t_1}{(k_2 + k')^2} \right) \left( 1 - e^{-\left( \frac{k_2 + k'}{V} \right) t_1} \right) - \frac{k_2 C}{k_1 V - k_2 - k'} \left( e^{-\left( \frac{k_2 + k'}{V} \right) t_1} - e^{-k_1 t_1} \right) - \frac{k_2 k_3}{k_2 + k'} t_1 \quad (12)$$

and for secretion —

$$C_2 = \left( \frac{k_2 C}{k'} + \frac{k_2 k_1 V}{(k')^2} \right) \left( 1 - e^{-\frac{k' t_1}{V}} \right) - \frac{k_2 C}{k_1 V - k} \left( e^{-\frac{k' t_1}{V}} - e^{-k_1 t_1} \right) - \frac{k_2 k_1}{k'} t_1 \quad (13)$$

It is interesting to note that if the concentration in the blood is falling rapidly, i.e.  $k_2$  is large compared with  $k'$  the concentration in the anterior chamber may reach or even exceed that in the blood, even though the equilibrium ratio is ordinarily below 1. Clearly, no quantitative inference as to the rate of transfer or mechanism involved can be drawn from observations that do not include continuous determinations of the concentration of the test substance in the blood, and treatment of the data which does not consider the existence of outflow from the anterior chamber.

As reported in the preceding paper the blood concentration in the urea experiments was maintained constant throughout the period of the investigation. However, urea differs from the other substances which will be analyzed quantitatively in that some is present initially in the anterior chamber, i.e.,  $C_2 \neq 0$  at  $t = 0$ , but  $= C_1$ . In this instance the integration is still carried out between the limits  $t = 0$  and  $t = t_1$  remembering, however, that at  $t = 0$ ,  $C_2 = C_1$  with the result that on the basis of the ultrafiltration hypothesis

$$C_2 = \frac{k_2}{k_2 + k'} \left[ C \left( 1 - e^{-\left( \frac{k_2 + k}{V} \right) t_1} \right) \right] + C_1 e^{-\left( \frac{k_2 + k}{V} \right) t_1} \quad (14)$$

On the basis of the secretion hypothesis

$$C_2 = \frac{k_2}{k'} C \left( 1 - e^{-\frac{k' t_1}{V}} \right) + C_1 e^{-\frac{k' t_1}{V}} \quad (15)$$

## RESULTS

Fig. 1 shows the result of calculating the coefficient of transfer ( $k_2$ ) for sodium from the experimental data (reported in (1)) for various assumed values of  $k'$  on the basis of the ultrafiltration hypothesis. Fig. 2 shows the results of analogous calculations made on the basis of the secretory hypothesis. The broken line, in each case, represents the values of  $k_2$  and  $k'$  which would be required by the ratio of concentration in the anterior chamber to that in the blood under equilibrium conditions. The value used, 0.90 was obtained by estimation from the sodium curve as shown in Fig. 1 of (1) and appears to be of the same order of magnitude as that reported by others from chemical studies.

It is at once apparent from Fig. 1 that a reasonable fit of the experimental data (measured concentration in the anterior chamber and in the blood) is found only when  $k_2$  is equal to about 3.5, and  $k'$  equal to something less than  $\frac{1}{2}$ . With two exceptions all of the ratios give values for  $k_2$  which vary only from 3.0 to 4.4 for this value of  $k'$ , whereas with slightly greater assumed rates of flow it may be seen that  $k_2$  varies tremendously for the different experimental points.

From Fig 2, it is clear that once again the best fit is found when  $k_2$  is about 3.5, but in this instance  $k'$  is almost 4.0

The results from the chloride experiments have been calculated in a similar manner and the plots are shown in Fig 3 for the ultrafiltration and secretion hypotheses. Since the equilibrium ratio for chloride is approximately 1.03 the slope of a line showing the relation of  $k_2$  to  $k'$  under equilibrium conditions would

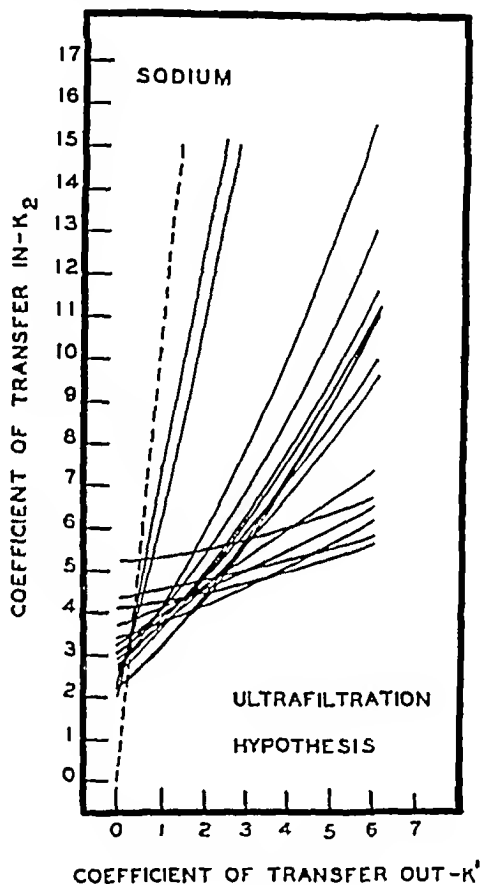


FIG 1

be negative on the basis of ultrafiltration, and since factors other than possible flow of aqueous out of the anterior chamber presumably would be needed to account for this slope, no line has been shown in case of ultrafiltration. However, the broken line on the set of curves representing secretion again represents this equilibrium ratio.

It is clear from Fig 3 that the best fit for the filtration hypothesis is given when little or no flow is present, a result similar to that found for sodium. In this instance  $k_2$  has an average value of 4.5. On the basis of the secretory hypothesis the best fit results when  $k_2$  is 3.9 and  $k'$  is 3.85.

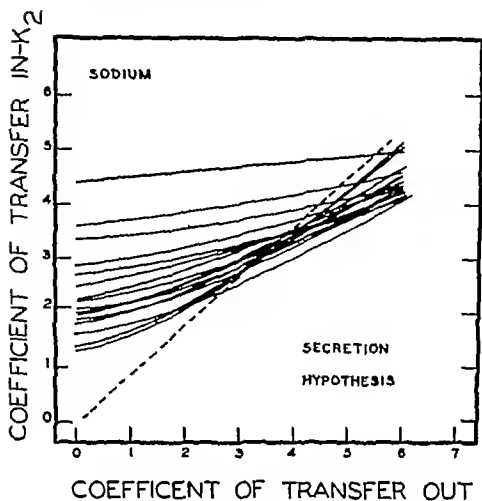


FIG. 2

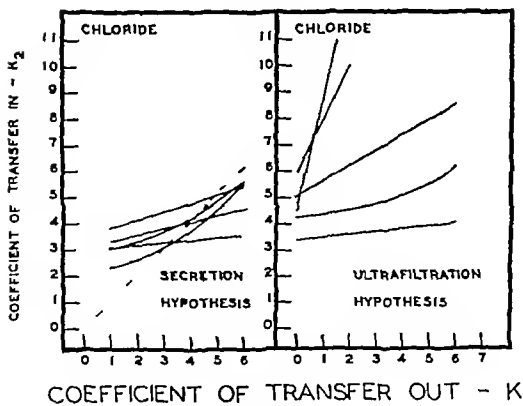


FIG 3

Similarly, the results from the SCN experiments are plotted and shown in Fig 4. On the basis of the ultrafiltration hypothesis it would appear that again the best fit is when  $k'$  is equal to zero, and the corresponding value of  $k_2$  is 3.8. The curves based on the secretory hypothesis are most similar at  $k_2 = 4.0$  and  $k' =$  approximately the same value.

In the case of lithium only two experimental points were available to compute the rate of transfer into the anterior chamber. Nevertheless it may be seen from Fig 5 that on the basis of ultrafiltration a fit is given only when  $k'$  is about zero. If the lithium enters by secretion, however,  $k'$  again is found to be equal

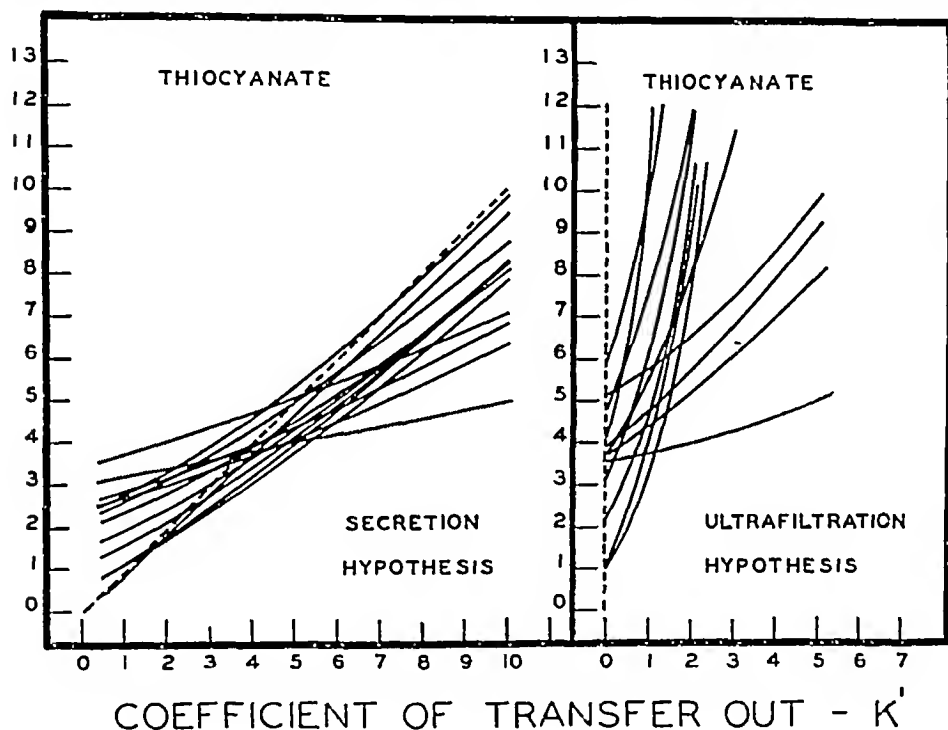


FIG 4

to approximately 4, and  $k_2$  in this instance appears to be about 3.5. The broken line is plotted on the assumption that the final equilibrium ratio of lithium in the aqueous to that in the plasma is 1.

From Fig 6 it may be seen that the best fit on the basis of ultrafiltration for bromide occurs when  $k_2 = 4.0$  and  $k' =$  zero, while  $k_2 = 4.0$  and  $k' = ca. 4$  if it be assumed that bromide enters the anterior chamber by a secretory process. Again the broken lines are based on an assumed equilibrium ratio of 1.

It will be recalled that except for minor differences two consistently different values (zero and 4) are obtained for the coefficient of outflow ( $k'$ ), depending upon whether one assumes that these electrolytes enter the anterior chamber by filtration or secretion. Since either set of values for the coefficients of transfer

$k_2$  and  $k'$  will account for the determined concentrations there is no way of distinguishing which hypothesis is the correct one. Moreover, while the rel-

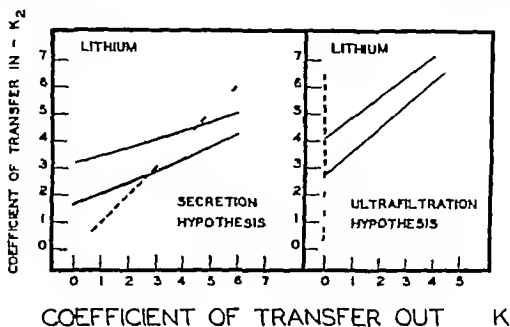


FIG 5

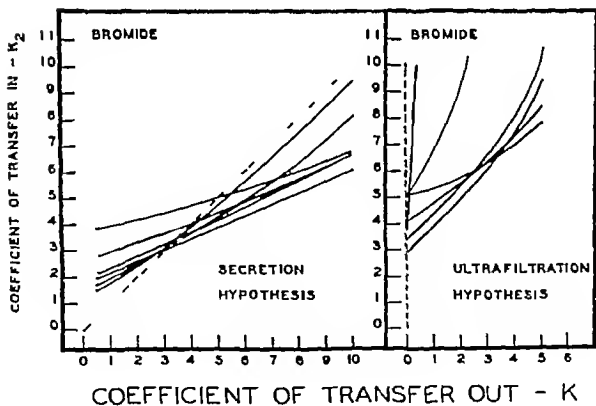


FIG 6

ative rates of transfer into the anterior chamber have now been determined the absolute rate too, depends to some extent upon one's assumption of the processes involved



If, for the present, we do not examine the evidence from other sources for a through and through circulation of aqueous, but first consider the general relationship between  $k_2$  and the equilibrium ratios required by the two hypotheses for various values of  $k'$ , and secondly, consider the case of substances which give rise to equilibrium ratios significantly less than 1, it will be seen that additional information is obtained which is helpful in deciding which mechanism is operative for transferring the substances tested into the anterior chamber

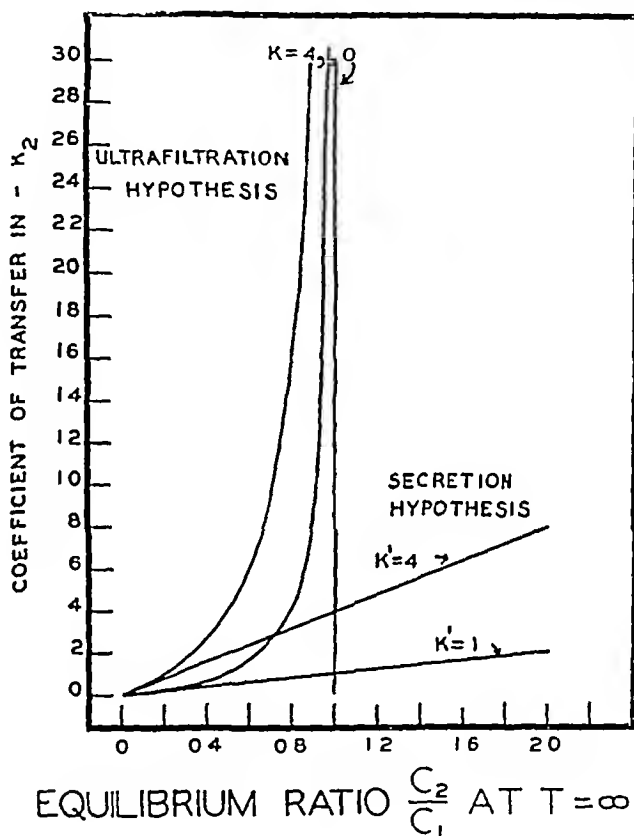


FIG 7

The relation between the equilibrium ratio and the coefficient of transfer out of the anterior chamber by means of a flow ( $k'$ ) on the basis of the ultrafiltration and secretory hypotheses may be seen from Fig 7. Here three values, 0, 1, and 4, have been assumed for  $k'$  and the equilibrium ratios have been calculated for various values of  $k_2$  from equations 3 and 6, where  $t$  equals infinity. The solid perpendicular line shows the expected ratio on the basis of the ultrafiltration hypothesis at  $k'$  equals 0. This calculation has not taken the Gibbs-Donnan equilibrium into account, but had this been done the line would still be perpendicular but would be shifted to the right or left slightly depending upon whether one were dealing with an anion or cation. On the basis of the

secretion hypothesis there would be no equilibrium for  $k'$  equal to 0, hence no line is shown. As was to be expected, equilibrium ratios significantly below 1 cannot be accounted for by either hypothesis under the assumed condition of  $k' = 0$ .

The two curved lines show the expected equilibrium ratios when  $k'$  equals 1 and 4 for the ultrafiltration and the two sloping straight lines show the analogous ratios for the secretory hypothesis.

The great increase in value of  $k_2$  required to produce an equilibrium ratio above 80 per cent on the basis of the ultrafiltration hypothesis, compared to that required by the secretion hypothesis suggests that on the knowledge of the rate of transfer of water and urea few substances would be found in the anterior

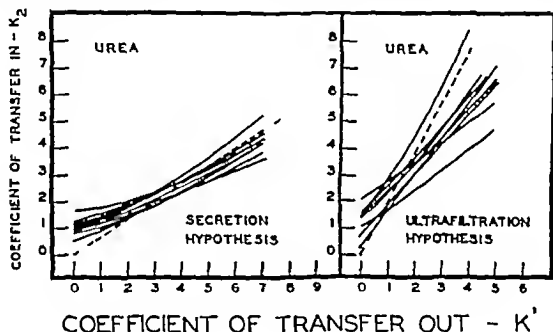


FIG 8

chamber having equilibrium ratios above this amount if they entered by means of filtration and any substantial loss occurred through flow.

Urea was one substance which gave an equilibrium ratio of less than 1. The coefficients of transfer in and out of the anterior chamber for this compound are shown plotted against each other in Fig 8. Again the broken line represents the equilibrium ratio (0.66). Unlike previous results on the basis of the ultrafiltration hypothesis the best fit is obtained when  $k'$  is more than zero—in this instance it would appear to equal approximately 1.7 and  $k_2 = 2.8$ . The corresponding values given by the secretion hypothesis are  $k' = 4.0$  and  $k_2 = 2.7$ . Thus, as was anticipated for all substances which show a deficiency in concentration in the anterior chamber the experimental data give a reasonable fit only when it is assumed that there is some leakage regardless of which of the two hypotheses is presumed to be operative.

Since it has been shown that concentrations of electrolytes found exper

mentally would not be attained by an ultrafiltration process if there is any substantial leakage by flow, but could be attained by a secretory process under such conditions, it would appear that all of the data are compatible only with the idea that some leakage does occur

If we assume that the  $k'$  value for urea is 4 and calculate the final equilibrium ratio which gives a good fit for the  $k_2$ - $k'$  plots on the basis of the ultrafiltration hypothesis it is found to be 58 per cent. While this value was closely approximated by 4 out of 5<sup>4</sup> of the last experimental points shown on Fig. 5 of the preceding paper it will be recalled that the average equilibrium value found in control animals was  $66 \pm 5$  per cent.

Considering the variability of urea ratios in both treated and untreated animals the authors are inclined to believe that the possibility of urea entering the anterior chamber by ultrafiltration cannot be discarded.

It will be recalled from Fig. 6 of the preceding paper that the levulose concentrations in the blood of different rabbits was too variable to permit representing them all with a single curve. For this reason it is not possible to analyze quantitatively the results in the manner used for the other test substances. Nevertheless, the fact that the maximum concentration reached in the anterior chamber is only about 35 per cent of that in the plasma seems indicative that either the excess levulose is quite rapidly metabolized<sup>5</sup> or that it too, may be lost from the anterior chamber as a result of a flow process.

If the coefficient of flow ( $k'$ ) is set equal to 4 and one solves for  $k_2$  on the bases of both hypotheses when the equilibrium ratio is 35 per cent,  $k_2$  is found to equal 2.15 for ultrafiltration and 1.4 for secretion. From a plot of the results obtained by substituting these values in Equations 14 and 15 and solving for the concentration ratios at various times, curves are obtained which produce a reasonably good fit for the aqueous curve of Fig. 6 of the previous paper.

Out of interest a similar calculation was made letting  $k' = 1.7$  (the result obtained in the case of urea-ultrafiltration hypothesis). The resulting curve in this instance was much flatter at the beginning than the others and does not produce as good a fit.

The phosphate results too (Fig. 4 in the previous paper) were so variable that

<sup>4</sup> The ratios were 60, 59.4, 68, 57.5, and 58.5 per cent for  $t = 81, 91, 115, 140$ , and 176 mins. respectively.

<sup>5</sup> The question of utilization has frequently been invoked to account for deficiencies in the anterior chamber without proof that it actually could quantitatively do so. Several years ago one of the authors (V. E. K.) in an *in vitro* experiment increased the amount of glucose available for rabbit lens utilization from 90 mg. per cent to twenty times this concentration and found that the oxygen uptakes were identical. While the glycolysis was not measured in these experiments it would appear that the oxygen uptake of the lens, at least, does not fluctuate significantly with concentrations of carbohydrate in excess of normal.

only a qualitative analysis seems warranted. It is certain however, that the ratio when equilibrium is obtained is definitely below 1, again the conclusion seems inevitable on the basis of present knowledge at least, that some leakage must occur to account for the findings. With regard to the actual rates of transfer into the anterior chamber, if  $k'$  is assumed to be 4, a value of  $k_2$  equal to 7.4 for the ultrafiltration and 2.6 for the secretion hypothesis would account for the observed equilibrium ratio of about 65 per cent.

From a consideration of the results obtained with urea, levulose, and phosphate it would appear that the ratios observed may be accounted for only on the assumption that there is a flow out of the anterior chamber. The exact magnitude of this flow may be open to some question but from the fact that a reasonably good fit to the experimental curves results if one substitutes a value of 4 as obtained from the other test materials, it seems justifiable to conclude that this value is substantially correct. One implication of such a conclusion is, as was suggested before, that the rate of leakage out by flow is the same for all of the compounds used. Obviously this may not be the case, but the fact that ions of sizes as different as Na and SCN appear to leave the anterior chamber at essentially the same rate suggests that for ions of this range of size at least, it is true. If, as is usually assumed, the flow occurs through Schlemm's canal, the walls, being composed of endothelium similar to that found in capillaries, would not be expected to differentiate between compounds no larger than levulose.

In any case so long as  $k'$  is substantially above zero the data for all of the electrolytes tested, except  $\text{PO}_4$ , give a good fit only on the assumption that they are secreted. Phosphate too, gives a fit on such an assumption but since an equally good fit is obtained assuming ultrafiltration, the choice in this instance cannot be made from the data at hand. Since the data for the compounds having equilibrium ratios less than one are not differentiable by any such conspicuous differences in  $k'$  values as was found for the electrolytes on the basis of the two hypotheses, the choice of which one best accounts for their passage from the blood to the anterior chamber must come from other sources.

#### DISCUSSION

That the units for the constants  $k_1$  and  $k'$  are in c. mm. per minute may readily be seen from Equation 1. It should be emphasized that the  $k_2$  values show the relative rates at which different substances can enter the anterior chamber under identical concentration conditions, in the plasma only, when the transfer is the result of a secretory process, and in the plasma and in the anterior chamber when the transfer is the result of ultrafiltration.

Since the concentration is different for each substance in the plasma the actual rate of transfer must likewise be different and is equal to  $k_2 C_1$  on the basis of secretion and  $k_2(C_1 - C_2)$  on the basis of ultrafiltration. Thus each

mentally would not be attained by an ultrafiltration process if there is any substantial leakage by flow, but could be attained by a secretory process under such conditions, it would appear that all of the data are compatible only with the idea that some leakage does occur

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Considering the variability of urea ratios in both treated and untreated animals the authors are inclined to believe that the possibility of urea entering the anterior chamber by ultrafiltration cannot be discarded.

It will be recalled from Fig. 6 of the preceding paper that the levulose concentrations in the blood of different rabbits was too variable to permit representing them all with a single curve. For this reason it is not possible to analyze quantitatively the results in the manner used for the other test substances. Nevertheless, the fact that the maximum concentration reached in the anterior chamber is only about 35 per cent of that in the plasma seems indicative that either the excess levulose is quite rapidly metabolized<sup>5</sup> or that it too, may be lost from the anterior chamber as a result of a flow process.

If the coefficient of flow ( $k'$ ) is set equal to 4 and one solves for  $k_2$  on the bases of both hypotheses when the equilibrium ratio is 35 per cent,  $k_2$  is found to equal 2.15 for ultrafiltration and 1.4 for secretion. From a plot of the results obtained by substituting these values in Equations 14 and 15 and solving for the concentration ratios at various times, curves are obtained which produce a reasonably good fit for the aqueous curve of Fig. 6 of the previous paper.

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the difference in osmotic activity. This difference amounted to an average of 5.4 mm of NaCl per kilo of water.

While our studies have shown that the rates of transfer into the anterior chamber of both anions and cations are compatible with a mathematical formulation based on a secretory process, it should be pointed out that active secretion of ions bearing only positive or only negative charges may occur. In such an instance the oppositely charged ion would of necessity be transferred into the anterior chamber in order to maintain electrical neutrality. This latter process would lead to concentrations indistinguishable from those which would be obtained had the active secretion applied to the other ion or for that matter to both ions.

If we are to accept the mechanism for ion transfer by secretion as propounded by Friedenwald and coworkers, and we are aware of no others, it would appear that it is the cation which is actively secreted into the anterior chamber and the anion is transferred as a result of electrical attraction. The transfer of non-charged substances would presumably occur, in the absence of experimental evidence to the contrary, by means of simple filtration. Suggestive of this is the penetration of neutral dyes through the stromal-epithelium barrier of the ciliary body, as shown by Friedenwald and Stiehler.

With the object of determining the effect of valence on the rates of transfer the substances chosen for study included mono-, bi-, and trivalent ions. For reasons already cited the results for the bivalent ion (Mg) and the trivalent one (Fe) could not be used, and while the phosphate lead to an equilibrium ratio significantly less than one the contribution of charge in establishing such an equilibrium ratio is complicated by the several ionic forms of phosphate having different valences.

It is interesting to note that the rates of transfer into the anterior chamber on the basis of the secretory hypothesis for all of the monovalent electrolytes tested were approximately the same irrespective of the size of the test substance. By contrast, the proportionately lower transfer rates found by us for urea and levulose and the progressively lower equilibrium ratios found by Weld (9) for glucose, sucrose, and raffinose are suggestive of the passive nature (ultrafiltration) of the process of transfer for non-charged molecules.

From the fact that a flow out of the anterior chamber produces concentrations in the aqueous humor lower than in the blood for all substances which enter by means of ultrafiltration and for any which enter by secretion having  $k_2$  values less than 4 ( $k_2$  less than the rate of flow), one would anticipate that some other substances would have  $k_2$  values in excess of 4, and thus be present (as a result of secretion) in higher concentrations than in the blood in order to maintain the osmotic equilibrium. Chloride appears to be one such substance. Moreover the osmotic equilibrium, hence the intraocular pressure, is continually dependent upon both the magnitude of the rate of flow and the rate of transfer

into the anterior chamber, particularly that part of the latter due to secretion. Any decrease in the rate of flow or increase in rate of secretion (or rate of ultrafiltration) would be expected to increase the intraocular pressure and *vice versa*. It is probable that in a normal eye an increase in pressure would augment the rate of flow, or perhaps lower the transfer rate. These compensatory changes would tend to minimize the rise in pressure.

The application of the mechanisms outlined in this paper to the problem of intraocular pressure and its relation to glaucoma will be discussed in a later paper.

#### SUMMARY

The importance of considering the effect of a possible flow out of the anterior chamber before inferring any mechanism of aqueous humor formation from the relative concentration of a substance in the aqueous humor and plasma under equilibrium conditions has been stressed.

Several processes to account for the chemical equilibria between aqueous humor and blood based on the ultrafiltration and secretion hypotheses with a possible simultaneous loss of aqueous humor by flow have been outlined. On the basis of these processes, equations were formulated which would relate the rates of transfer into and out of the anterior chamber to the ratio of concentration of a substance in the aqueous to that in the blood at various intervals after its introduction into the blood. The explanation of equilibrium ratios above and below one for aqueous constituents is made apparent from the mathematical formulations. For each substance tested a determination was made of the best fit when the concentration in the aqueous humor is plotted against time. This fit was obtained by plotting the rate of transfer in against the rate of transfer out of the anterior chamber for all of the experimentally found concentration ratios on the basis of both the ultrafiltration and secretory hypotheses. Two sets of values were obtained from these calculations, one set for each hypothesis.

The substantial agreement of all the experimental data with an assumed rate of leakage out of the anterior chamber of approximately 4 c mm per minute was shown to be compatible only with the idea that all the monovalent electrolytes tested entered the anterior chamber as a result of secretory process. It could not be decided from these chemical studies whether the non-electrolytes and the one multivalent electrolyte tested enter the anterior chamber by ultrafiltration or secretion.

Experimental findings from other sources were cited which would suggest that non-electrolytes enter the anterior chamber as a result of ultrafiltration.

The implications of the mechanism outlined in the paper with respect to intraocular pressure have been discussed.

Supplementary evidence from the literature has been given in support of the conclusions presented here.

The authors wish to thank Dr Elek Ludvig and Dr Ransom Lynch for their aid in the formulation and integration of the mathematical expressions and the former for many helpful criticisms of the work.

They are also indebted to Miss Cynthia Stertz who gave technical assistance in these studies

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into tyrosinase) 0.3 ml of 0.4 per cent tyramine hydrochloride was placed in the side bulb of each flask. After equilibration in a water bath at 24.9°C the contents of the flasks were mixed. The reciprocal of the time for the uptake of the initial 100  $\mu$ l of oxygen was found. This specific reaction velocity for that analysis of the control in the presence of sodium dodecyl sulfate was given the value of unity. Differently treated portions of the same extract were compared on the basis of the relation which their specific reaction velocities bore to that of the control.

## RESULTS

The set of curves in the upper part of Fig. 1 describes the attainment of equilibrium among protyrosinase, tyrosinase, and inactive products at various pH values from 4.40 to 10.60. The upper curve in each member of this representative set pertains to the sum of the relative amounts of protyrosinase and tyrosinase which exist during exposure to any one pH. Each of the lower curves is determined by the relative amount of tyrosinase which is produced by the pH treatment. When either both curves or the upper curve decline toward the abscissa, as at pH 10.60 or 4.40, it seems that inactive products are being formed in the one case from tyrosinase and protyrosinase and in the other from protyrosinase alone. After some 3,000 minutes, equilibrium values seem to be attained. Curves A and D (Fig. 1), constructed from a total of 24 such equilibrium values, may be called pH stability diagrams of protyrosinase. The left hand limb of curve A portrays the shift of protyrosinase into inactive products, whereas the right hand limb from pH 9.30 to pH 10.34 records the inactivation of both protyrosinase and tyrosinase. There seems to be a mixture of tyrosinase and protyrosinase at the latter hydrogen ion concentrations, since the ascending and then descending parts of curve D record the shift of protyrosinase into tyrosinase and thence into inactive products. The activation of protyrosinase by treatment with high hydroxyl ion concentrations seems to be irreversible. A sample of protyrosinase which had been exposed to pH 10.08 for 1460 minutes showed no reversion of tyrosinase to protyrosinase for so long as some 3,000 minutes at 25°C after lowering the pH to 6.72 by adding phosphate buffer.

Since a tyrosinase results from exposure of protyrosinase to high hydroxyl ion concentrations, it seems necessary to see whether this "new" tyrosinase resembles that produced by other methods. Its thermolability and sensitivity to cyanide and diethyldithiocarbamate were therefore tested. This tyrosinase was destroyed by heating at 90°C for 5 minutes. It was completely poisoned by  $1 \times 10^{-4}M$  cyanide or diethyldithiocarbamate. These characteristics proved to be identical with those of tyrosinase produced by the action of sodium dodecyl sulfate.

The pH stability diagram of heat produced tyrosinase is indicated by curves B and C which were constructed according to the procedure described in the legend of Fig. 1. Because of some heat inactivation, curve B, throughout most

of its course, is at a lower level than curve A. Although the heat treatment produced 0.6 tyrosinase (curve C), it also resulted in the destruction of 0.2 of

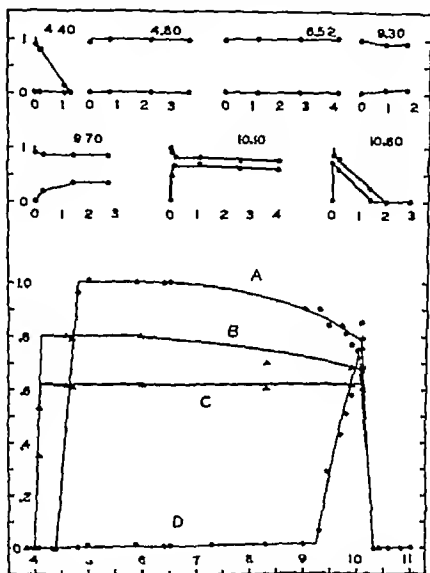


FIG. 1 The effect of exposing protyrosinase to various hydrogen ion concentrations at 25°C. The ordinates are the ratio of the specific reaction velocity to that of the control tube. The abscissae for the upper set of curves are given in thousands of minutes. The abscissa for the lower curves is given in pH units. Each pair of the upper curves was constructed from data of the analyses performed on protyrosinase which had been exposed to the indicated pH. The upper member of each pair shows the activity obtained in the presence of sodium dodecyl sulfate, whereas the lower member shows the activity in the absence of dodecyl sulfate. Equilibrium values from the upper members were used in constructing curve A. Similar values from the lower members were used for curve D. The foregoing procedure was also used in constructing curves B and C for heat produced tyrosinase.

the mixture of protyrosinase and tyrosinase (curve B). The right hand limbs of curves B and C apparently coincide with those of curves A and D. This coincidence is probably due to the fact that in these cases only tyrosinase was



# STUDIES ON THE METABOLISM OF THE AUTOTROPHIC BACTERIA\*

## III THE NATURE OF THE ENERGY STORAGE MATERIAL ACTIVE IN THE CHEMOSYNTHETIC PROCESS

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(Received for publication June 29 1942)

The most striking result of a study of the process of chemosynthesis (synthesis of all materials from  $\text{CO}_2$  using chemical energy obtained by oxidation) in the autotrophic bacterium, *Thiobacillus thiooxidans*, was the ability of the cell to store up the energy derived from sulfur oxidation in an available form which could be used for  $\text{CO}_2$  fixation under conditions in which sulfur oxidation was impossible (Vogler, 1942). This ability to store the energy of sulfur oxidation in the cell seems to differentiate the process of chemosynthesis from that of photosynthesis except that definitive experiments in photosynthesis are not yet available. It was of considerable interest to determine the nature of this readily available storage product. The data contained in this paper show that the oxidation of sulfur is directly coupled to transfers of inorganic phosphorus from the medium to the cells. They also show that  $\text{CO}_2$  uptake is coupled to transfers of inorganic phosphate from the cell to the medium and that  $\text{CO}_2$  fixation is dependent, in the absence of concomitant sulfur oxidation, upon the amount of phosphate previously taken up during sulfur oxidation. These relationships strongly suggest that the energy storage product is a phosphorylated compound and that the energy is stored in the cell as phosphate bond energy (cf. Lipmann, 1941). Such a finding relates the process of energy storage and transfer in the autotrophic bacterium to that of heterotrophic organisms (cf. Kalckar 1941, Lipmann, 1941).

### EXPERIMENTAL

It was observed in several experiments that *Thiobacillus thiooxidans* does not take up or release significant amounts of inorganic phosphate during sulfur oxidation by resting cells in the presence of  $\text{CO}_2$ . During respiration in the absence of sulfur there were small changes in the level of inorganic phosphate in the medium and during prolonged endogenous respiration considerable phosphate appeared in the medium. It was then found that during sulfur oxidation in the absence of  $\text{CO}_2$ , inorganic phosphate was taken up by the cells. These occurred in such a manner that they might be related to the chemosyn-

\* This work was supported by the Wisconsin Alumni Research Foundation

thetic process and with the development of accurate methods for estimating the  $\text{CO}_2$  fixation (Vogler, 1942) and phosphate (O'Kane, 1941) it became possible to study more closely this phosphate metabolism. A number of experiments were done. Ultimately, however, the several factors involved must be studied simultaneously. An experiment in which this was done is illustrated in Fig. 1, based upon the data of Table I which also includes other information

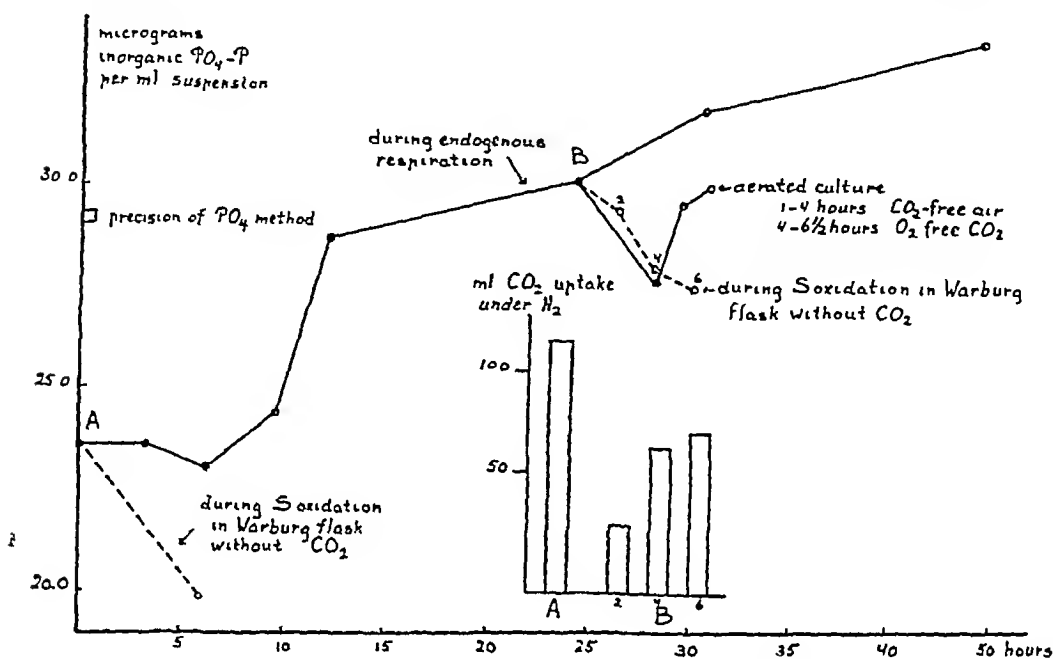


FIG. 1 Phosphate changes during respiration and  $\text{CO}_2$  fixation by *Thiobacillus thiooxidans*. See text.

which it is not convenient to illustrate in the figure. The experiment is described briefly below.

**Experiment I**—To a suspension of 8 day old cells in distilled water,  $\text{KH}_2\text{PO}_4$  was added to obtain a final concentration of 23.7 micrograms phosphorus per ml. 100 ml of this suspension (containing 168 micrograms nitrogen per ml as bacterial cells) were aerated with air for 44 hours. Samples of 4 ml were removed at intervals, mixed with 1 ml trichloroacetic acid (100 per cent), and centrifuged. In the supernatant fluid inorganic phosphate was determined on duplicate 2 ml samples by the method of Fiske and Subbarow (O'Kane, 1941). This method uses the Evelyn photoelectric colorimeter and determines orthophosphate in amounts of from 10 to 80 micrograms with a minimum precision of 0.4 micrograms.

On samples of 3 ml drawn at the zero hour (point A in Fig. 1) and after 24 hours aeration (point B, Fig. 1) the endogenous respiration and the  $\text{CO}_2$  uptake were determined. After 24 hours aeration three samples of 5 ml were placed in Dixon-Keilin flasks and 1 ml of a sulfur suspension was added per flask.

To 20 ml. of the remaining stock suspension 4 ml. sulfur suspension was added and the aeration was continued with CO<sub>2</sub>-free air. During this treatment inorganic phosphate disappeared from the medium until after 4 hours a sample was taken and

TABLE I

*Phosphorus Changes during Respiration of Thiothrix thiooxidans*

Aeration in the absence of sulfur

Hours	Inorganic P per ml. <i>micrograms</i>	Standard deviation	QO <sub>2</sub> (N) in absence of sulfur	CO <sub>2</sub> uptake in H <sub>2</sub> <i>μl.</i>	Samples
0	23.7	0.1	8.2	15.0	A
3	23.7	0.1			
6	23.0	0.0			
9	24.5	0.1			
12	29.4	0.1	2.0	0.0	B
24	30.0	0.1			
30	31.9	0.1			
36	32.4	0.0			
44	33.6	0.1			

20 ml. sample removed at B plus 4 ml. sulfur suspension

Time	Treatment	Hours	Inorganic P	Standard deviation
24	Air no CO <sub>2</sub>	0	30.0	0.1
28	Air no CO <sub>2</sub>	4	27.7	0.1
29	CO <sub>2</sub> only	5	29.5	0.0
30	CO <sub>2</sub> only	6½	30.0	0.1

Samples in Dixon Keilin flasks

Gas Phase		Flask 1 5 ml. sample B + 1 ml. sulfur suspension	Flask 2 as flask 1	Flask 3 as flask 1	Flask 4 5 ml. sample A + 1 ml. sulfur suspension
Air	Time of oxidation	2 hrs.	4 hrs.	6 hrs.	6 hrs.
	CO <sub>2</sub> uptake	220 μl.	640 μl.	1070 μl.	1650 μl.
	Initial PO <sub>4</sub> -P	30.0	30.0	30.0	23.7
	Final PO <sub>4</sub> -P	29.5	28.0	27.5	19.8
	PO <sub>4</sub> -P uptake	0.5	2.0	2.5	3.9
H <sub>2</sub>	CO <sub>2</sub> uptake	32 μl.	72 μl.	80 μl.	125 μl.

All data on phosphorus in terms of micrograms phosphorus per 168 micrograms bacterial nitrogen. Oxygen uptake and CO<sub>2</sub> uptake recorded per 168 micrograms bacterial nitrogen.

\* Standard deviation of the mean.

CO<sub>2</sub>-free air was replaced with a stream of O<sub>2</sub>-free CO<sub>2</sub>. Samples then were taken after 1 hour and after 2½ hours (solid line from point B, Fig. 1).

The three samples in Dixon Keilin flasks were placed in the Warburg apparatus and at intervals the O<sub>2</sub> uptake was determined and a 3 ml. sample drawn from one

flask for a phosphate determination. This flask was then placed back in the Warburg and its atmosphere replaced with hydrogen. After 1 hour, when the lack of uptake indicated the absence of  $O_2$ ,  $CO_2$  was added in known amounts.  $CO_2$  uptake occurred in all flasks. The amount of  $CO_2$  uptake under hydrogen is shown in the bar graph of Fig. 1.

The addition of the sulfur suspension in the early part of the experiment altered the concentrations of cells and phosphate. All data on phosphate have been calculated back, however, to the original suspension of 168 microgram N/ml. The data used in the figure plus certain other information that cannot be recorded conveniently in the graph are given in Table I.

The data show that endogenously the cells released phosphorus in an inorganic form after the first 6 hour period during which no change was apparent. When the suspension was supplied with sulfur and aerated with  $CO_2$ -free air (solid line-point B) there was an uptake of inorganic  $PO_4$ . When oxygen was removed, however, and replaced by pure  $CO_2$  (from 28 to 31½ hours) this  $PO_4$  was again released. It was released rather rapidly, most of it appearing in the 1st hour, which correlates well with the observations in the previous paper (Vogler, 1942) which show that most of  $CO_2$  uptake in the absence of sulfur occurs in the 1st hour. There is thus a strong indication that this phosphate release is connected with  $CO_2$  synthesis which is occurring in the absence of oxygen.

Even more significant are the data obtained in the Warburg flasks. At point A the suspension placed on sulfur took up 1650  $\mu l$   $O_2$  (per 168 micrograms cell nitrogen) over a period of 6 hours in the absence of  $CO_2$  ( $Q_{O_2}(N) = 1635$ ). During this time 3.9 micrograms of phosphorus (per 168 micrograms cell nitrogen) were taken up as well. After 24 hours aeration (at point B) the suspension took up 220, 640, and 1070  $\mu l$   $O_2$  (per 168 micrograms cell nitrogen), after 2, 4, and 6 hours, respectively, on sulfur in the absence of  $CO_2$ . During these periods 0.5, 2, and 2.5 micrograms phosphorus were taken up. That is, in both of these cases during sulfur oxidation (in the absence of  $CO_2$ ) phosphate was taken up by the cells. When these cells were placed in an atmosphere of hydrogen free from oxygen (so that there could be no further oxidation of the sulfur) and  $CO_2$  was supplied, the  $CO_2$  uptake was proportional to the phosphate that had been taken up previously. The actual figures show that close to 32  $\mu l$   $CO_2$  were taken up per microgram of phosphorus. Since 22.4  $\mu l$   $CO_2$  is equal to 1 micromol, and since 31 micrograms of phosphorus equal 1 micromol, the molar ratio of  $CO_2/PO_4 = 44$ . The data on phosphorus uptake in this series, however, are really not suitable for such calculations since the suspensions were heavy. As a result only a small part of the cells was attached to sulfur whereas the majority were in suspension and continued to liberate phosphate. This probably accounts for the low phosphate uptake observed during the first 2 hours (point B) and also explains the rapidly in-

creasing rate of  $O_2$  uptake ( $Q_{O_2}(N)$  at point A = 1635, at B, 2 hours 655, 4 hours 950, 6 hours 1060)

There is one point that needs re-emphasis, namely, that the  $CO_2$  uptake in the absence of oxygen is proportional to the  $PO_4$  taken up during the previous oxidation and not to the time through which this oxidation occurred. For example, in the samples taken at point B, there was not much difference between those oxidations taking place for 4 or 6 hours with respect to either  $PO_4$  or  $CO_2$  taken up. It therefore appears as though there were a limited amount of the energy storage product and that under the physiological conditions existing at B, this became almost saturated in 4 hours. It is apparent that the energy storage product formed during sulfur oxidation is related to the phosphorus changes.

It has previously been mentioned (Vogler *et al.*, 1942) that under the conditions of our experiments most of the cells are in contact with the sulfur particles when the concentration of bacterial nitrogen is 20 micrograms per ml. ( $Q_{O_2}(N)$  becomes constant at a high level). Since the method of phosphorus determination is most accurate over a range between 10 and 70 micrograms (precision 0.4 micrograms) and since the amount of phosphorus exchanged is of the order of 3 to 4 micrograms phosphorus per 200 micrograms of bacterial nitrogen, large samples of dilute suspensions are necessary for phosphorus determinations. Moreover, a determination of the percentage of the cells attached to the sulfur is necessary. This latter may be accomplished by measuring the turbidity of the fluid after the sulfur (and attached organisms) have settled and is most conveniently expressed in terms of bacterial nitrogen. An experiment conducted with these principles in mind gave results as follows.

*Experiment II*—A suspension of 8 day old cells in a phosphate solution was diluted so that it contained 20 micrograms bacterial nitrogen per ml. and adjusted to 20 micrograms per ml. phosphate. This suspension was kept overnight for 12 hours at room temperature in order to allow any diffusion effects to come to equilibrium. 60 ml. of this suspension were then pipetted into 124 ml. Warburg flasks, and sulfur was added. Aliquots of 10 ml. were withdrawn at the intervals indicated in Table II for determinations of inorganic phosphate, and cells not attached to sulfur. 1 ml. trichloroacetic acid (100 per cent) was added after the turbidity due to unattached cells had been determined and the samples were kept in the refrigerator until the experiment was complete when inorganic phosphate was determined on all samples simultaneously.

The sulfur settled out to the bottom of the flask when the flasks were not shaken; the samples therefore were virtually free from sulfur particles which might interfere with the determinations and they contained only cells suspended in the medium, but none attached to sulfur particles.

Sulfur oxidation was measured within 20 minutes after the sulfur had been added. The  $O_2$  uptake during those 20 minutes may be disregarded. After sulfur oxidation had continued for 3 hours the flasks were aerated with  $H_2$  for 1 hour then with  $N_2$ .



for another hour. The absence of  $O_2$  was apparent from the lack of uptake. Known amounts of  $CO_2$  were then added to give a concentration of about 2 per cent  $CO_2$ . A control containing no sulfur was carried through the same procedure.

It is apparent (Table II) that some time after the 1st hour most of the cells became attached to the sulfur since the nitrogen content of the supernatant fluid (measured by cell turbidity) dropped from the original 20 micrograms per ml to 3.5 micrograms.

During the 1st hour there was accordingly no uptake of phosphate and little of oxygen. During the next 2 hours the cells attached themselves to the sulfur and from the average amount of cells attached ( $16.5/2 = 8.3$  micrograms bacterial nitrogen per ml), one can calculate that the average  $Q_{O_2}(N)$  of the cells over this period was 1253.

TABLE II

*Phosphorus Changes during Respiration and Chemosynthesis by Thiobacillus Thiooxidans*

Time	Vol	Gas phase	With sulfur gas uptake	N per ml	P per 10 ml	Without sulfur microgram P per 10 ml
hrs	ml		$\mu l$	micrograms	micrograms	
0	60	Air, no $CO_2$	—	20.0	33.7	33.7
1	50	"	160 $O_2$	20.0	34.1	—
3	40	"	1002 $O_2$	3.5	30.2	34.0
5	20	$H_2$ 1 hr	0	2.5	29.9	34.0
		$N_2$ 1 hr				
7	20	$N_2$ 2 per cent $CO_2$	200 $CO_2$	1.2	32.9	34.2

During the next 2 hours, in which there was no oxygen available, there was no gaseous uptake and very little phosphate change (0.3 micrograms per 10 ml) which is about the limit of the analytical method and which could be accounted for by the oxygen uptake occurring before the hydrogen has entirely replaced the air. As soon as all the oxygen was removed, there was no further uptake indicating that hydrogen is inactive in this organism. In order to be sure that the hydrogen was not combining with the  $CO_2$  it was replaced by nitrogen before the  $CO_2$  was added, although in other cases this replacement was not necessary and hydrogen appears to be as inert as nitrogen for this organism. During this period a few more organisms attached themselves to the sulfur. These probably played no part in the phosphate changes since they had no opportunity to replenish their phosphate or energy stores even in the presence of sulfur since there was no oxygen available for oxidation.

From the results of both experiments it is apparent that

1. During sulfur oxidation in the absence of  $CO_2$ , inorganic phosphate is taken up by the cells. It thus appears that the sulfur oxidation is coupled with the phosphate intake.

2 During  $\text{CO}_2$  fixation  $\text{PO}_4$  is released in an amount proportional to the  $\text{CO}_2$  fixed. It thus appears that the  $\text{CO}_2$  fixation is coupled with the release of phosphate

From this information one is able to conclude that the energy reservoir accumulated in the cell by sulfur oxidation and usable for  $\text{CO}_2$  fixation if  $\text{CO}_2$  is available, is a phosphorylated compound (or compounds)

### *Qualitative vs Quantitative Interpretations*

We have so far confined our attentions to the qualitative aspects of the phosphate exchange. These we feel are justified since we are working with intact living cells, in which the phosphate exchanges are not stoichiometrically related to energy or even to phosphate transfers because sources of phosphate other than inorganic within the living cell may be changed from one energy level to another without apparent change in the phosphate contents of the cell or surrounding medium. However, it is possible to show that there is a quantitative relationship between the phosphate and the sulfur oxidation and between the phosphate and the  $\text{CO}_2$  fixation. Both experiments show that it takes about 70 to 80 mols of oxygen to take up 1 mol of phosphate and that the release of 1 mol of phosphate fixes from 40 to 50 mols of  $\text{CO}_2$ . For example in Experiment II during the time of oxidation 21  $\mu\text{l}$ . of  $\text{O}_2$  were taken up per ml of suspensions and 0.39 microgram of phosphorus disappeared giving a molar  $\text{O}_2/\text{PO}_4$  ratio of 74. Similarly 10  $\mu\text{l}$ . of  $\text{CO}_2$  were fixed per ml and 0.3 microgram  $\text{PO}_4\text{-P}$  was released giving a molar  $\text{CO}_2/\text{PO}_4$  ratio of 46. If these ratios be calculated upon the basis of the number of organisms actually in contact with sulfur they are slightly modified to  $\text{O}_2/\text{PO}_4 = 72$   $\text{CO}_2/\text{PO}_4 = 47$ .

Such ratios are useful in two ways. First from them one can calculate the  $\text{O}_2/\text{CO}_2$  ratio (1.58:1.53) which can be compared with the ratio available from thermodynamic calculations (1.49). In this case therefore, it appears that a little more oxygen has been used than should have been thermodynamically necessary, but the efficiency appears very high. These calculations are included to show that even on a quantitative basis there are relationships between the  $\text{O}_2$ , the  $\text{CO}_2$ , and the  $\text{PO}_4$  which bear a reasonable resemblance to what would be expected. At the present state of our knowledge we cannot be certain that the values are real. The agreement between the thermodynamic and experimental values may be entirely fortuitous. For example one cannot be certain from the data available that the autotrophic bacterium has converted the fixed  $\text{CO}_2$  into carbohydrate which is the compound used for calculating the thermodynamic ratios.

A second use for these ratios is the determination of the phosphate bond energy (cf Lipmann 1941). If it requires 72 mols of  $\text{O}_2$  for each mol of  $\text{PO}_4$  then the phosphate bond would contain 5 688 000 calories since each mol of oxygen can liberate 79 000 calories of free energy from the oxidation of sulfur. If 47 mols  $\text{CO}_2$  are synthesized to carbohydrate by 1 mol  $\text{PO}_4$  the phosphate bond energy would be 5 952 000. The energy rich phosphate bonds in heterotrophic tissues contain ca 10 000 calories and there is no reason to believe that the ones produced here are any different. The high energies calculated therefore indicate that the phosphate changes measured are only a portion of those which actually occurred. This is due to two factors. One, since the measurements are made on the inorganic phosphate in

surrounding the cell, and since this organism is not sensitive to acid (it will respire for almost an hour in 5 per cent trichloroacetic acid) the permeability of the cell membrane becomes an important factor. One can, upon the basis of the assumption of a Donnan equilibrium, calculate the "real" phosphate changes, but these calculations involve so many assumptions that at the present time it does not appear worth while to include them. Two, there is evidence for a whole series of phosphorylated compounds in the autotroph (O'Kane, 1941) and the phosphate energy carrier formed during sulfur oxidation would, in all probability, be in equilibrium with these other esters. Its formation during sulfur oxidation would result in a shift in the concentrations of a number of phosphorylated compounds, all of which are not detected in the changes in inorganic phosphate. Attempts to measure these other compounds have been limited by the lack of suitable micro-methods. The phosphate taken up during sulfur oxidation is thus probably passed on (at least in part) to other phosphorus compounds and is probably not immediately released as inorganic phosphate during  $\text{CO}_2$  fixation. In fact the data suggest that the phosphate uptake occurs after the active phosphorus compounds in the cell have been raised to a high energy level. The rate of uptake at point B (Fig. 1) for 2 hours and 4 hours is suggestive of this phenomena. Also, the cells at point A (Fig. 1), although already containing the energy carrier (as evidenced by their ability to fix  $\text{CO}_2$  in the absence of sulfur, see Table I), still had the capacity to take up further phosphate, which is in accord with the conception outlined above. Many of the phenomena concerned with young and old cells are readily explainable upon this basis. The breakdown of organic storage products occurs only when the depletion of the energy carrier compounds requires a shift in the equilibrium of the whole system and proceeds only as far as is necessary to establish the overall equilibrium. Thus, "young" suspensions, being high in energy carrier compounds, have a very low endogenous respiration. During sulfur oxidation energy-rich phosphate bonds are accumulated resulting in a general increase in phosphorylated compounds and a concomitant increase in the energy level of many cell constituents as well as an increase in their amounts. Johnson (1941) has employed a somewhat similar concept for the explanation of the Pasteur effect in heterotrophic tissues.

### *Phosphate Inhibition*

The previous sections have indicated that the energy from the sulfur oxidation can be converted to energy of phosphorylation. This does not go on indefinitely, however, and only a certain amount of phosphate is taken up. Yet, in the Warburg flask, sulfur oxidation goes on for hours at a constant rate in the actual absence of  $\text{CO}_2$ . While it is conceivable that the energy built up in the form of the phosphorylated energy compound could be used in other cell activities, e.g. synthesis of nitrogenous compounds, etc., in the absence of  $\text{CO}_2$  and available nitrogen these synthetic actions could not account for a continued sulfur oxidation. It is therefore difficult to see how sulfur oxidation could continue at a constant rate in the absence of carbon dioxide.

One possible explanation of this experimental fact would be that the energy of phosphorylation is continually dissipated by the phosphatases of the cell

and that these agents therefore permit continued sulfur oxidation. It is sometimes possible to inhibit phosphatases by means of inorganic phosphate. The inhibition is thought to be due to the competition between the substrate and the phosphate for the enzyme. If the breakdown of the phosphorylated energy storage material by phosphatases is the explanation of continued sulfur oxidation in the absence of carbon dioxide, it should be possible to inhibit sulfur oxidation by increasing quantities of phosphate when carbon dioxide is absent but sulfur oxidation should not be so inhibited in the presence of carbon dioxide.

TABLE III  
*The Inhibitory Effect of Phosphate on Sulfur Oxidation*

Flasks containing 3 ml. suspension of 20 micrograms bacterial nitrogen per ml.	Before addition of CO <sub>2</sub> QO <sub>2</sub> (N)		CO <sub>2</sub> added	CO <sub>2</sub> taken up	O <sub>2</sub> taken up	Total uptake	QO <sub>2</sub> + QCO <sub>2</sub> (N)
			$\mu$ l.	$\mu$ l.	$\mu$ l.	$\mu$ l.	
2 M KH <sub>2</sub> PO <sub>4</sub>	20	0	184	74	7	81	670
0.2 M KH <sub>2</sub> PO <sub>4</sub>	640	380	168	103	181	284	2380
0.02 M KH <sub>2</sub> PO <sub>4</sub>	700	720	238	121	174	295	2480
0.0002 M KH <sub>2</sub> PO <sub>4</sub>	1640	1640	206	29	250	279	2320
	30-90 min.	180-240 min.	Over 2 hr. -period 240-360 min.				
	(1)	(2)	(3)	(4)	(5)	(6)	(7)

*Counteracting Possible Osmotic Influences*

Flasks containing the same suspension

	QO <sub>2</sub> (N)	
0.5 M KH <sub>2</sub> PO <sub>4</sub> + 0 M MgSO <sub>4</sub>	225	210
0.1 M KH <sub>2</sub> PO <sub>4</sub> + 0.4 M MgSO <sub>4</sub>	740	970
0.02 M KH <sub>2</sub> PO <sub>4</sub> + 0.48 M MgSO <sub>4</sub>	760	1060
0.004 M KH <sub>2</sub> PO <sub>4</sub> + 0.496 M MgSO <sub>4</sub>	960	1170
	0-30 min.	450-510 min.

*Experiment III*—Young cells with a low level of endogenous respiration (suspension harvested from an 8 day old culture) were suspended with sulfur in solutions of KH<sub>2</sub>PO<sub>4</sub> of different concentrations at pH 4.8. Each flask contained 3 ml. suspensions containing 20 micrograms of bacterial N/ml. The QO<sub>2</sub>(N) values were obtained with KOH in the center cup from 30 to 90 minutes after the cells had been suspended in the phosphate solution and in the 3rd hour after that time. Immediately thereafter strong H<sub>2</sub>SO<sub>4</sub> was added to neutralize the KOH in the center cups. CO<sub>2</sub> was added to each flask in a known amount and the uptake of CO<sub>2</sub> and O<sub>2</sub> measured during the next 2 hour period.

The data obtained are contained in Table III.

It is apparent that the rate of sulfur oxidation is inhibited by higher phosphate concentration, as was postulated in the previous discussion. Two molar  $\text{KH}_2\text{PO}_4$  was perhaps introducing other effects, but the lower quantities show the effect clearly (particularly column 2). If  $\text{CO}_2$  be supplied, however, which provides an alternative use for the energy storage material, the phosphate inhibition is overcome and the total uptake (column 6) is virtually constant. After  $\text{CO}_2$  had been added approximately equal quantities of this gas were taken up in presence of phosphate (column 4) and far less in the suspension containing only 0.0002 M phosphate (cells without added  $\text{PO}_4$ ).

Since it is possible that the observed effects might be due to osmotic relations, an experiment was done in which the osmotic pressure was held constant by additions of  $\text{MgSO}_4$  (lower part of Table III) but the same effects were noted (*i.e.*, lower sulfur oxidation with increased phosphate).

We therefore feel that the continued oxidation of sulfur in Warburg flasks in the absence of  $\text{CO}_2$  is due to the continual release of inorganic phosphate by the phosphatases in the cell. These can be inhibited by increases in the phosphate content of the medium which thereby inhibits sulfur oxidation. If  $\text{CO}_2$  is supplied it serves as an alternative use for the  $\text{PO}_4$  coming from sulfur oxidation and relieves the phosphate inhibition. This provides further evidence that  $\text{PO}_4$  is concerned both in sulfur oxidation and in  $\text{CO}_2$  fixation.

#### SUMMARY

In the autotrophic bacterium, *Thiobacillus thiooxidans*, the oxidation of sulfur is coupled to transfers of phosphate from the medium to the cells.  $\text{CO}_2$  fixation is coupled to transfers of inorganic phosphate from the cells to the medium and is dependent, in the absence of concomitant sulfur oxidation, upon the amount of phosphate previously taken up during sulfur oxidation. The energy reservoir, which is formed by sulfur oxidation in the absence of  $\text{CO}_2$  and which can be released for the fixation of  $\text{CO}_2$  under conditions which do not permit sulfur oxidation, is a phosphorylated compound and the data suggest that the energy is stored in the cell as phosphate bond energy. It is possible to oxidize sulfur at a constant rate for hours in the absence of  $\text{CO}_2$ . The phosphate energy formed during this process is probably released by cell phosphatases. It is possible to inhibit these phosphatases by means of inorganic phosphate and thus to inhibit sulfur oxidation in the absence of  $\text{CO}_2$ . In the presence of  $\text{CO}_2$ , where alternative uses for the phosphate energy are available, the inhibition is relieved. Sulfur oxidation (energy input) is coupled, not to  $\text{CO}_2$  fixation, but to phosphate esterification.  $\text{CO}_2$  fixation (energy utilization) is coupled with phosphate release.

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# THE USE OF A MEASURABLE CAUSE OF DEATH (HEMORRHAGE) FOR THE EVALUATION OF AGING\*

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## INTRODUCTION

Investigation of the problem of aging has been handicapped by the lack of a "yardstick" for evaluating the process of aging. *Chronological age* is an unsatisfactory standard because of the large variation between individuals and because it does not exclude irrelevant changes or secondary effects of aging. As was previously stated (1), "the increasing death rate appears to be the only manifestation of aging which, during adult life, shows wide changes that are subject to accurate measurement." Hence some measure of the acceleration of death rate should be useful in the study of aging.

*Longevity* (either mean or maximum life span) has been used as a measure of aging. However, longevity depends upon at least three other variables: first, the intrinsic death rate ( $P_0$  in equation 1) from those diseases which eventually kill the animals; second, the rate of increase in mortality with age ( $k$  which in turn, depends not only upon the aging process but also upon the individual variability) and, third, upon any influence which the experimental treatment may have upon specific diseases (change in  $P_0$ ). A given treatment may increase the experimental life span without influencing the rate of aging. Hence longevity is not a true criterion of aging.<sup>1</sup> Moreover, experiments on longevity require years for completion.

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<sup>1</sup> An equation for longevity can be obtained from equation (1a) by substituting  $-dN/N dt$  for  $P_t$  and integrating. This gives us

$$k_{10}t = \log \left[ \frac{k_1}{P_0} \log_{10} \left( \frac{N_0}{N_1} \right) + 1 \right] \quad (2)$$

This may be converted into logarithms to the base 10 (where  $k_{10} = 0.434 k$ )

$$k_{10}t = \log_{10} \left[ \frac{5.3 k_{10}}{P_0} \log_{10} \left( \frac{N_0}{N_1} \right) + 1 \right] \quad (3)$$

(Footnote continued on following page)





periment because it is a death involving the vascular system and one which can be regulated and measured. The technique was carefully planned, then subjected to initial tests and improvements before the data herewith reported were obtained.

### *Method of Bleeding*

Rats must not be subjected to rough handling or disturbing noise on the day they are to be bled. A cage containing a litter of rats of known age is brought gently into the room and allowed to stand without disturbance for an hour or more before the bleeding is started.

One of the rats is then gently placed in a 3 liter beaker having a cover. A wad of cotton (about 3 gm.) is treated with about 10 ml. of ether and dropped into the beaker. When the rat is completely anesthetized it is fastened to the bleeding board shown in Fig. 1. The rat's legs are held out with loops of heavy cotton string attached to 4 inch lengths of brass ladder chain which are hooked over brads on the board and can be easily adjusted. The head is held still by means of a wire loop attached to ladder

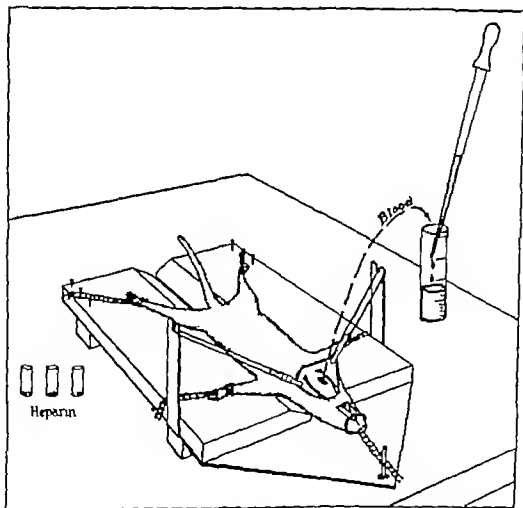


FIG. 1. Rat during bleeding. The right jugular vein is shown severed with blood flowing from the distal portion. The board is  $8\frac{1}{2}$  by 12 inches. It has a trough 2 inches wide by  $\frac{1}{4}$  inch deep and has a triangular piece of metal (for which wood could be substituted) at the front to hold the post to which the rat's head is hitched.

chain The loop fits over the rat's head and is caught behind the upper fangs A small wad of cotton, saturated with ether, is placed near the rat's nose to maintain an anesthesia just sufficient to prevent struggling

The skin over the throat is slit longitudinally with scissors and the flaps are pulled up, by means of clamps and elastic bands, to form a pocket<sup>3</sup> If the jugular veins are hidden by fibrous tissue this is gently dissected to expose the veins and to permit free flow of blood

Dry heparin (about 25 mg) is then sprinkled in the pocket and on the vein which is to be cut With small scissors one jugular vein is then severed at a point as proximal to the heart as possible At the moment of cutting the assistant presses a stop watch or notes the time in order to have a record of the duration of bleeding A rapid flow of blood is obtained at first and this blood is quickly mixed with the dry heparin by the assistant using a pipette with a rubber bulb (similar to a long medicine dropper) The assistant then transfers the blood, by means of the pipette, from the pocket to a weighed tube capable of holding 15 to 20 ml A graduated centrifuge tube can be used (and weighed by suspending in a wire ring of known weight, preferably 1 000 gm) A weighing bottle can be used but a graduated vessel gives the worker a better opportunity to gauge the progress of the bleeding A graduated vial is shown in Fig 1

The initial rapid flow may last 1 0 to 1 5 minutes and may yield 3 to 6 ml, or more, of blood By manipulation of the vein with iris forceps an attempt is made to keep the blood flowing slowly but constantly so that the rat will die in 8 to 10 minutes This is not always easy The vein often closes at its open end and it is necessary to cut off a small portion of the distal vein More heparin is always sprinkled on the tissue before a vein is cut and it is well to have, already prepared, three or four small tubes each containing about 25 mg of powdered heparin

After several minutes it may no longer be possible to obtain more blood from the first vein The other jugular vein is then severed, first proximally, then toward the head, as necessary If both veins fail to yield enough blood to kill the animal in 12 to 15 minutes one of the carotid arteries is cut This can be reached by pushing aside the muscles close to the clavicle However, the vagus stimulation (whether the vagus is severed or whether it is merely dissected away from the artery) is apt to stop the heart at this stage and only 2 or 3 drops of blood will be obtained Hence, it is best to avoid cutting the carotid arteries

The animal is not considered dead until the time when both the respiration and heart have stopped and remain stopped for at least 2 minutes afterwards It is not important that the exact moment of death be known The important information is the amount of hemorrhage required to produce death The time of bleeding is kept within certain limits merely to standardize the conditions

After death the blood is weighed ( $W_b$ ) and the animal is weighed ( $W_a$ ) The weight of the animal before bleeding is obviously equal to the sum of these two values Hence the "per cent hemorrhage," or "grams blood per 100 grams body weight" is equal to

$$\frac{100 W_b}{W_a + W_b} = 100 \frac{\text{Weight of blood}}{\text{Original weight of rat}}$$

<sup>3</sup> A similar technique, forming a pocket in a xilla of mice, was reported by Kuhn (3)

TABLE I

## Data on Bleeding of "Normal" Untreated Rats

The original weights and calculations were carried out to four significant figures, the last figure is omitted in this table.

Rat No.	Age	Weight	Blood per 100 gm body weight	Standard deviation	Standard error
	days	gm.	gm. per cent		
32	51	128	3.91		
33	51	141	4.12		
34	51	175	4.49		
35	51	119	4.33		
36	50	103	4.42		
37	50	100	4.42		
38	50	117	4.51		
39	50	106	4.47		
60	50	118	4.50		
61	50	112	4.02		
Mean	50 days	122 gm.	4.235 per cent	$\pm 0.23$	$\pm 0.07$
20	100	257	4.15		
46	100	218	3.76		
47	100	307	3.83		
48	100	236	3.91		
49	100	279	3.80		
50	100	221	4.01		
51	100	231	4.07		
62	100	234	3.78		
63	100	227	3.67		
64	100	223	4.03		
65	100	254	4.23		
66	100	246	3.76		
Mean	100 days	243 gm.	3.92 per cent	$\pm 0.19$	$\pm 0.05$
6	355	376	3.57		
7	355	389	3.84		
8	354	400	3.80		
9	355	464	3.81		
11	361	311	3.56		
12	361	330*	4.17		
13 F	357	232	3.66		
15 F	358	233*	4.11		
41	359	449	3.56		
42	359	426	3.59		
43	355	390	3.70		
44	355	385	3.65		
45	355	364	4.44		
Mean	355 days	379 gm.	3.69 per cent	$\pm 0.11$	$\pm 0.04$
24	644	374	3.78		
25	644	475	3.77		
34	626	471	3.83		
35	626	330	3.05		
Mean	635 days	418 gm.	3.62 per cent	$\pm 0.23$	$\pm 0.19$
26	827	257	3.12		
27	827	266	3.42		
28	826	425	3.06		
29	826	328	3.41		
30	826	419	3.59		
31	821	345	3.35		
32	821	353	3.70		
33	821	351	3.32		
Mean	824 days	346 gm.	3.435 per cent	$\pm 0.26$	$\pm 0.09$

\* Values discarded.

F means female. All others were males.

chain The loop fits over the rat's head and is caught behind the upper fangs A small wad of cotton, saturated with ether, is placed near the rat's nose to maintain an anesthesia just sufficient to prevent struggling

The skin over the throat is slit longitudinally with scissors and the flaps are pulled up, by means of clamps and elastic bands, to form a pocket<sup>3</sup> If the jugular veins are hidden by fibrous tissue this is gently dissected to expose the veins and to permit free flow of blood

Dry heparin (about 25 mg) is then sprinkled in the pocket and on the vein which is to be cut With small scissors one jugular vein is then severed at a point as proximal to the heart as possible At the moment of cutting the assistant presses a stop watch or notes the time in order to have a record of the duration of bleeding A rapid flow of blood is obtained at first and this blood is quickly mixed with the dry heparin by the assistant using a pipette with a rubber bulb (similar to a long medicine dropper) The assistant then transfers the blood, by means of the pipette, from the pocket to a weighed tube capable of holding 15 to 20 ml A graduated centrifuge tube can be used (and weighed by suspending in a wire ring of known weight, preferably 1 000 gm) A weighing bottle can be used but a graduated vessel gives the worker a better opportunity to gauge the progress of the bleeding A graduated vial is shown in Fig 1

The initial rapid flow may last 1 0 to 1 5 minutes and may yield 3 to 6 ml, or more, of blood By manipulation of the vein with iris forceps an attempt is made to keep the blood flowing slowly but constantly so that the rat will die in 8 to 10 minutes This is not always easy The vein often closes at its open end and it is necessary to cut off a small portion of the distal vein More heparin is always sprinkled on the tissue before a vein is cut and it is well to have, already prepared, three or four small tubes each containing about 25 mg of powdered heparin

After several minutes it may no longer be possible to obtain more blood from the first vein The other jugular vein is then severed, first proximally, then toward the head, as necessary If both veins fail to yield enough blood to kill the animal in 12 to 15 minutes one of the carotid arteries is cut This can be reached by pushing aside the muscles close to the clavicle However, the vagus stimulation (whether the vagus is severed or whether it is merely dissected away from the artery) is apt to stop the heart at this stage and only 2 or 3 drops of blood will be obtained Hence, it is best to avoid cutting the carotid arteries

The animal is not considered dead until the time when both the respiration and heart have stopped and remain stopped for at least 2 minutes afterwards It is not important that the exact moment of death be known The important information is the amount of hemorrhage required to produce death The time of bleeding is kept within certain limits merely to standardize the conditions

After death the blood is weighed ( $W_b$ ) and the animal is weighed ( $W_a$ ) The weight of the animal before bleeding is obviously equal to the sum of these two values Hence the "per cent hemorrhage," or "grams blood per 100 grams body weight" is equal to

$$\frac{100 W_b}{W_a + W_b} = 100 \frac{\text{Weight of blood}}{\text{Original weight of rat}}$$

<sup>3</sup> A similar technique, forming a pocket in a villa of mice, was reported by Kuhn (3)

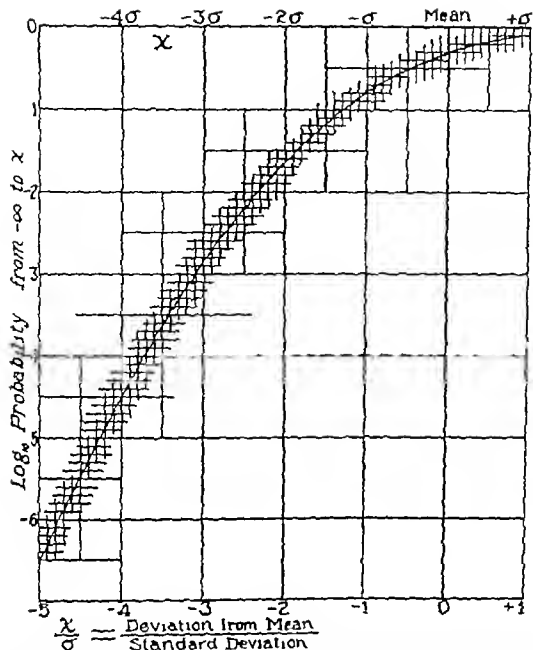


FIG 3 Curve for the logarithm of 'the normal probability integral' (i.e. the area under a normal distribution curve from  $-\infty$  to  $x$ ) The ordinates represent the logarithm of the probability of death when the hemorrhage is equal to  $x$  or less

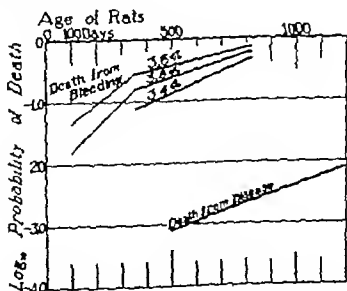


FIG 4 Computed mortality curves from hemorrhage. The slopes are essentially the same as that of the curve for spontaneous deaths from disease.

## RESULTS

In Table I the results obtained with 47 "normal" rats which were all in apparently good health (except for three of the oldest animals<sup>4</sup> which, however, gave values close to the mean for their group)

In Fig 2 the values are plotted. It will be seen that in rats older than 100 days the amount of hemorrhage required to produce death drops with age, following an essentially straight line curve. Because the young rats (50 days) obviously deviated considerably from those over 100 days, it would seem that up to 100 days the rats undergo "developmental changes" as distinguished from the subsequent "aging changes."

The 824 day rats died with a degree of hemorrhage 12 per cent less than was required to kill the 100 day rats. The 12 per cent change over a period of 724 days may seem small compared with the tenfold (1000 per cent) increase in rat mortality from disease during the same age range. However, the bleeding is expressed as quantity, whereas the mortality is expressed as probability.

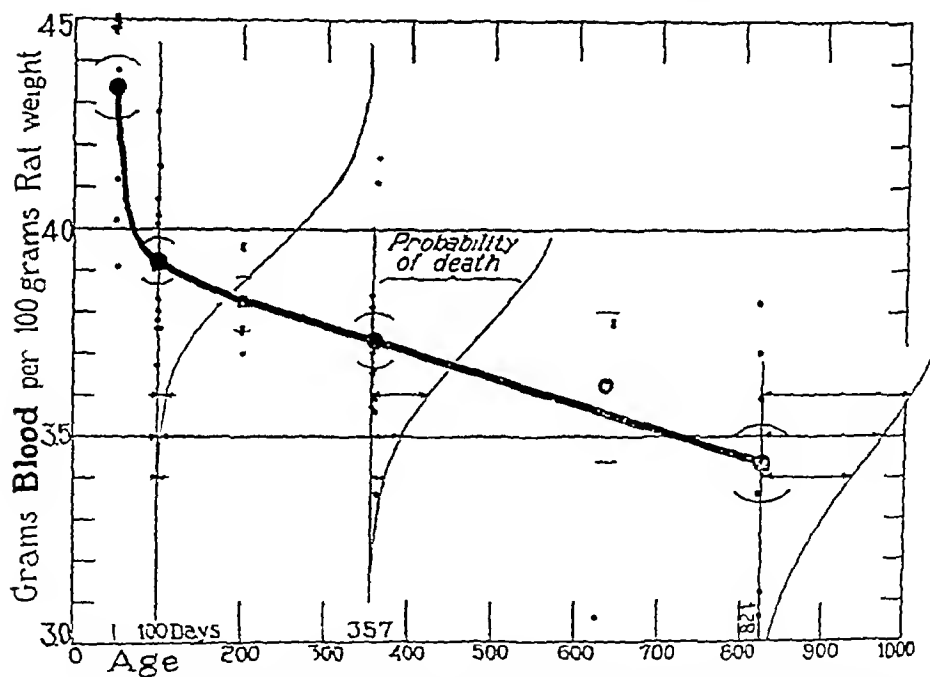


FIG 2 Bleeding data of rats. Small dots are individual values. Large dots are averages in each age group. The broken circles have radii equal to the standard error. The S-shaped curves are "probability integral" curves drawn relative to the mean values and based upon the standard deviation in each case.

<sup>4</sup> Rat 27 had a 2 cm chronic ulcer on its right axilla. Rat 29 had severe eczema and rat 30 had on its neck a cyst containing 20 ml of clear fluid.

more (d) the individual values can be correlated with autopsy findings on the changes in various tissues. It is to be expected that any change which involves the underlying process of aging should give better correlation with individual bleeding values than with chronological age.

Thus death from a known measurable cause may serve as a yardstick in determining what observable changes constitute the process of aging, and what treatments may alter the rate of aging.

### *Application of Method*

*Effect of Underfeeding*—As an illustration of the application of this method data are presented in Table II on several rats which were given a restricted diet by L. Pomerantz of the Department of Pharmacology. They were less than half normal weight. The hair was thin and they appeared asthenic. However, their bleeding values were normal for their age as may be seen in Fig 1 where the square dots at 200 days give an average value which lies on the line for normal animals. As far as can be concluded from so few animals the restricted diet had not affected their aging.

TABLE II  
*Rats Underfed for 84 Days by L. Pomerantz*

Rat No.	Age	Weight	Blood per 100 gm. body weight	Standard deviation	Standard error
	days	gm.	gm. per cent	"	M
36 F	(200)	127	3.75		
37 F	(200)	132	3.70		
38 F	(200)	132	3.95		
39 F	(200)	153	3.76		
40 F	(200)	140	3.96		
Mean	(200) days	137 gm.	3.82 per cent	±0.12	±0.06

F means female. The ages are accurate within 6 days.

### DISCUSSION

Any attempt to predict the results of future use of this method in the study of aging would be unwarranted. Present circumstances as well as the scarcity of old animals will delay adequate work along this line. Since aging is our most urgent medical problem (1) it is hoped that the use of this 'yardstick' will facilitate its study. It should be pointed out that for adequate investigations using this method, large continuous colonies of rats should be maintained under as favorable conditions as possible, subjecting the animals to no rough handling or disturbing noise.

The question may be raised as to what extent experimental error affects the bleeding values. Apparently this error is relatively small since any correction



Nevertheless the bleeding values can be converted into probability of death as follows. The standard deviation for each age group is calculated and with this value we can read from the curve in Fig 3 (see also the S-shaped curves in Fig 2) the logarithm of the probability that a rat of a given age will die from a given degree of bleeding or less. Thus for 3.6 per cent bleeding we may calculate that the probability of death for a 100 day rat is 0.045 while that for an 824 day rat is 0.73, or 16 times as great. Hence the 12 per cent difference in bleeding level between these two groups is magnified into a 1600 per cent difference when converted into probability of death at a given level.

In Fig 4 it will be seen that when these results are plotted as logarithms of probability of death, curves are obtained which approximate the slope of the curve for spontaneous deaths of rats from disease. Thus the linear physiological alteration (in resistance to hemorrhage) results in a logarithmic mortality curve. This is partly because the log probability integral curve in Fig 3 is essentially linear within a limited range.

### *Significance of Results*

The above results are significant in that

1 By an artificial known and measurable cause of death applied to normal animals of known age, we have reproduced the type of mortality curve found for spontaneous deaths from disease. Hence, in so far as the increase in death rate with age results from an underlying process of aging, this process of aging appears to have been involved in the cause of death which was used (hemorrhage).

2 The large logarithmic increase in death rate with age is shown to result from (a) a relatively small linear change in resistance to the cause of death, combined with (b) the effect of random individual variability. These two components can be separately evaluated. The change in resistance to the cause of death is small and linear (12 per cent change in the bleeding value) but involves the "underlying aging process." The variability appears to change little with age but the combined effect of the two components (a and b) results in a large (16-fold) change in death rate with age. The mortality curve is logarithmic partly because the probability integral approximates a logarithmic curve (see Fig 3) within a limited range.

3 As a tool in the study of the process of aging this technique offers hopeful possibilities.

(a) The cause of death can be altered by varying the bleeding technique, or by using asphyxia, anoxia, various toxins, etc. (b) The animals can be treated in various ways previous to the bleeding (administration of various drugs, hormones, diets, or removal of endocrines or other tissues, etc.) (c) The individual bleeding levels can be correlated with degree of physiological functions (blood volume, basal metabolism, kidney function, etc.) Further-

# A NEW METHOD FOR THE GENERATION AND USE OF FOCUSED ULTRASOUND IN EXPERIMENTAL BIOLOGY

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## INTRODUCTION

Ultrasonic, or supersonic waves as they are sometimes called, are mechanical vibrations in solid, liquid, or gaseous mediums lying above the range of human hearing. They have been produced mechanically and electrically, utilizing magnetostriction and piezo-electric effects. The latter method is the only one which is adapted to the generation of ultrasound at frequencies above 500 k.c., which is the range most often used in biological work. It consists of passing a high frequency electric current through a quartz plate, so that the latter expands and contracts in resonance with the alternations of the current passing through it, and emits ultrasound waves of a corresponding frequency.

The biological effects of supersonic waves were first thoroughly studied by Wood and Loomis (1) in 1926-27 at Tuxedo Park, New Jersey. They observed its stimulating and lethal effects on unicellular organisms, tissues, small fish, and animals. Since then, a host of subsequent investigators have expanded our knowledge regarding the thermal, chemical, and photochemical effects of ultrasound. Its dispersive power, its ability to produce stable emulsions between immiscible fluids such as mercury and water, and its stimulating and destructive effects on virus, bacteria, potato shoots, and animal tissues *in vitro* and *in vivo* have also been studied.

To date, all biological and neurological applications of supersonic waves have utilized plane waves, proceeding in parallel paths from the flat surface of the quartz crystal generator, and no attempt has been made to increase their local intensity by bringing them to a focus. However, some years ago, this possibility was suggested by the late Dr. James Chiles of the University of Virginia.

### *Purposes of the Present Investigation*

We have attempted to apply biologically the physical discovery of Grutzmacher (2) (1935) that very short ultrasonic waves can be focused by giving a concave curvature to the surface of the vibrating quartz plate, constituting the source of radiation. Thus, he was able to concentrate approximately 150 times as much ultrasonic energy at the focal spot as could be found at a similar spot close to the vibrating plate.

for experimental error would make the logarithmic mortality curves steeper than they are in Fig 4. While it is to be expected that deaths from a vascular cause, such as hemorrhage, should give a slightly steeper curve, nevertheless the order of magnitude is about as expected. Furthermore litter mates gave such surprisingly close agreement (note rats 56 to 60)<sup>5</sup> that the error could not have been great.

While we have spoken of hemorrhage as a "known cause of death" we do not know the actual mechanism of death even from this cause. The blood volume is reduced until the large veins tend to collapse and the large arteries are visibly smaller. The peripheral circulation is reduced until the feet are very pale and cyanotic. Death, as determined by blood flow, occurs with the cessation of heart action rather than from respiratory failure (one rat's heart continued to beat several minutes after respiration stopped but the reverse was never observed).

Whether the animals died from "shock" depends upon one's definition of that term, but since we do not know the mechanism of death from shock the point is not important. It was observed that the hematocrit fell during the bleeding, which would indicate the mobilization of fluid from the tissues.

The degree of bleeding was calculated relative to body weight rather than to total blood volume. It is hoped that in the future we may be able to determine the hemorrhage in terms of blood volume.

#### SUMMARY

1 There has been need for a reliable experimental standard for the process of aging. Chronological age is a poor standard owing to irrelevant changes and individual variability. Longevity does not indicate the rate of aging because it depends not only upon aging but also upon intrinsic death rate, individual variability, and the selective effect of experimental treatment upon resistance to specific diseases.

2 The use of a known measurable cause of death (hemorrhage) on healthy animals of various ages reproduces the known mortality curve for rats, and differentiates this measure of the aging process from individual variation.

3 The possibilities for the use of this method in determining the nature of the aging process are pointed out.

#### REFERENCES

- 1 Simms, H. S., *Science*, 1942, 95, 183
- 2 Simms, H. S., *Science*, 1940, 91, 7
- 3 Kuhn, L. R., *Science*, 1941, 95, 504

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<sup>5</sup> However, it was found that the last rat in each litter often gave values either higher or lower than any of his litter mates (note rat 61 as compared with Nos. 56 to 60). This erratic behavior was ascribed to the mental disturbance caused by finding himself alone for the first time in his life.

The master crystal control oscillator (Fig 1) utilizes an ordinary receiver type triode (type 56 or 76 depending on the filament heater voltage available) This is loosely coupled with the grid of the tube in the buffer stage

The buffer stage utilizes the R.C.A. 807, a newly developed beam power tube with high power sensitivity This provides the grid driving power necessary for Class C operation of the power amplifier T 200 tube on the top shelf (Figs 1 and 3)

The radio-frequency voltage to the ultrasonic crystal is controlled by varying the d.c. voltage to the plate of the power amplifier tube. This is accomplished by a voltage dropping variable resistor (Fig 1  $R_4$ ) To prevent initial transient voltages while turning on the circuit, from becoming excessive the best procedure was to put all this resistance in the circuit before turning on the T 200 plate voltage Then the resistance may be decreased until any predetermined radio-frequency ammeter reading (R.F.) is reached A spark gap (S G) is included across the tuned output circuit to take transient voltages that might otherwise injure the crystal. For long tube life resistor  $R_4$  must be at maximum when the crystal or other load is not coupled to the power amplifier otherwise the available power for this efficiently adjusted stage may have to be dissipated entirely in the anode of the tube

Resistor  $R_3$  acts as a 'safety valve' to limit the current to the plate which if overloaded too often may result in the tube current becoming erratic due to gases given off from overheated parts As the power amplifier draws more and more current the voltage drop across  $R_3$  automatically increases to put a higher negative voltage on the grid of the T 200 tube. Such protection is valuable when tuning adjustments are made or in case the master oscillator crystal should fail to oscillate, or in the event that anything should stop the driving power to the grid of the power tube. Resistors  $R_3$  and  $R_4$  are adjusted with peak load for the maximum radio-frequency current to the supersonic crystal

In the R.F. output circuit fixed condensers  $C_3$  bypass the alternating current but isolate the supersonic crystal and its container from the high voltage direct current on the plate of the power amplifier tube Not only do these condensers provide safety for the operator but they also help to neutralize the inductance of the long leads to the supersonic crystal.

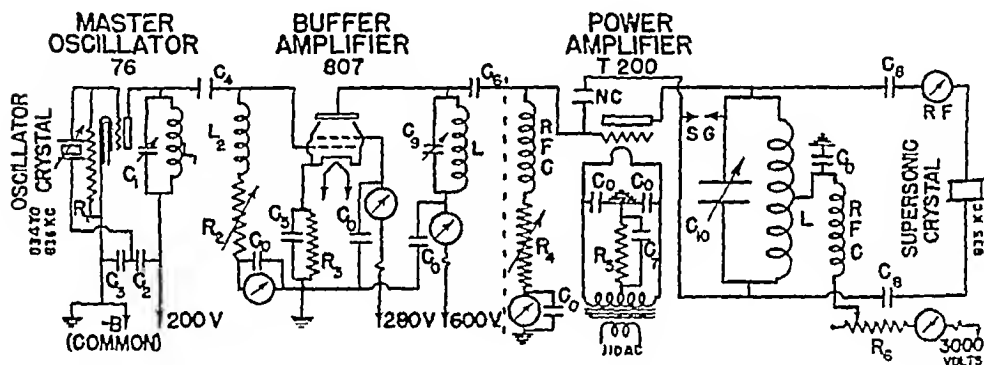
In the construction of a radio-frequency generator of this kind, compactness of parts and efficiency usually go hand in hand All radio-frequency connections should be made with short heavy wires Care was taken to have all ground for the buffer stage connect to one common centrally located point on the chassis which was in turn grounded to a water pipe. Tuned circuits were adjusted for minimum plate current or just a little past this point for stability An outline of the proper procedure for neutralizing and tuning a master oscillator power amplifier such as this one is given in the Radio amateur's handbook obtainable in any library

The chief feature of the foregoing design is the use of a quartz crystal as a master oscillator instead of an inductance and a capacity in the first grid circuit. The final ultrasound generating crystal is ground to match this oscillator crystal, so that the entire unit may be tuned to resonance and neutralized once and for all. 'Fishing' for the supersonic crystal frequency is unnecessary A vernier adjustment, built into the master oscillator crystal holder allows a very slight frequency shift (834-836 k.c.) so

The chief aim of the present study has been to project such a focused beam of supersound into fresh tissue blocks and into the tissues and organs of living animals, so as to produce a maximum of change deep at the spot of focus with a minimum of change in the intervening tissues traversed by the beam before it reaches the focus. While much more work has still to be done towards determining the optimum frequency, exposure time, intensity, etc. necessary to produce such focal effects in deep lying tissue, yet sufficient progress has been made in developing an efficient focusing ultrasound generator and in applying it to biological and neurological material to justify this early report.

### *The Radio-Frequency Power Generator*

The oscillating electrical potential across the opposite faces of the x-cut concave quartz crystal, ground to a natural frequency of 835 kc, is supplied by what amounts, in principle, to a small  $\frac{1}{2}$  kw radio transmitter, such as is used for code signalling. Both a rear view photograph, Fig 3, and a schematic diagram, Fig 1, are given.



lite plunger which can be moved back and forth so as to vary the depth of the ultrasonic focus in the material irradiated

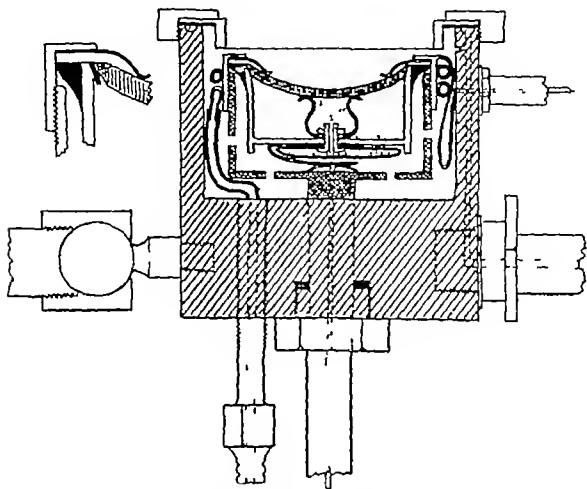


FIG 2 Diagram of the ultrasonic generator This is housed in a transparent bakelite container (diagonal lines) supported by a ball and socket joint (lower left) The container is sealed above by a cellophane diaphragm against which biological specimens are placed its interior is filled with transformer oil (clear) in which the curved quartz crystal vibrates The latter is mounted by its beveled edge on a neoprene gasket (black) attached to the margins of a bakelite cup The interior of this cup forms an air filled chamber (stipple) behind the crystal Electrical leads (top right and bottom center) connect with spring contacts on the two aluminized faces of the curved crystal The nodal suspension of the crystal by its beveled edge is shown in detail (upper left) Only one of the two water conduits (bottom left) is shown connected via neoprene tubing to the copper cooling tube on the outside of the brass electrode collar A device for sucking out air bubbles under cellophane diaphragm is shown at lower right Neoprene gaskets spreaders and air expansion bag are indicated in solid black

The entire generator is immersed in transformer oil contained in a heavy walled transparent bakelite cylinder The oil is electrical insulating but ultrasound transmitting Together they are efficient insulators against both the R.F current and stray ultrasonic waves The oil transmits the focused beam of supersound to a thin

that it is possible to set it at exact resonance with the driven ultrasound crystal (835 kc) This gives a maximum power transfer, which, together with the accurate and stable means of tuning, accounts in part for the high supersonic output obtained with the relatively low radio-frequency power available

### *The Focusing Ultrasound Generator*

A special crystal mounting, holder, and container had to be designed to meet the unusual requirements incidental to the most efficient operation of a curved crystal from which focused ultrasound could be applied in any selected direction, to a predetermined depth, in the tissues of living animals (Fig 4) Only innovations and improvements of design will be described here Standard practice is followed in many details which are thoroughly described in *Ultrasonics* by Bergmann (3)

The ideal crystal mounting must provide a firm support yet allow the quartz to vibrate freely with a minimum of restraint at the edges or dampening of the surfaces Both of these factors reduce ultrasonic output, and, in addition, restraint of the edges produces distortion with a tendency for the crystal to crack Bechmann (4) partially solved the latter problem and reduced dampening by freely mounting the vibrating quartz on three pointed ivory pins which contacted the crystal at the nodal line along its grooved edge Oyama (5), Grutzmacher (6), and Dognon, Dognon, and Biancani (7) utilized an air chamber behind the crystal This not only reduced dampening effects, but also acted as a reflector of high-frequency ultrasound, so as to more than double the sonic output from the other side Technical difficulties have hitherto precluded the simultaneous adoption of the advantages of both mountings, but in the present design, their difficulties have been overcome and both the nodal line suspension of the quartz and the reflecting airchamber are successfully combined in the same mounting (Fig 2)

Oil-impervious synthetic rubber and fairprene rubber cement have been utilized in mounting the focusing crystal along the nodal line of its beveled edge This served the dual function of giving a firm but elastic mounting of a high dielectric character and also of providing an efficient gasket which sealed off the air chamber behind the crystal In order, however, to allow for expansion of heated air in this chamber, a neoprene expansion bag was attached to its base (Fig 2)

The crystal and its mounting are surrounded by transformer oil and held by three neoprene spreaders in the center of a thin-walled bakelite cup or holder with perforations in the bottom which allow for the free circulation of insulating oil into the space between the side walls of the mounting and those of the bakelite holder This bakelite holder is also provided with a wide screw-on brass collar to which is soldered water-cooled copper coils Thus, this broad-surfaced brass collar acts both as an oil cooler and as one electrode with spring extensions contacting the outer aluminized surface of the crystal The holder with its contents is mounted on the end of a laminated bake-

lite plunger which can be moved back and forth so as to vary the depth of the ultrasonic focus in the material irradiated

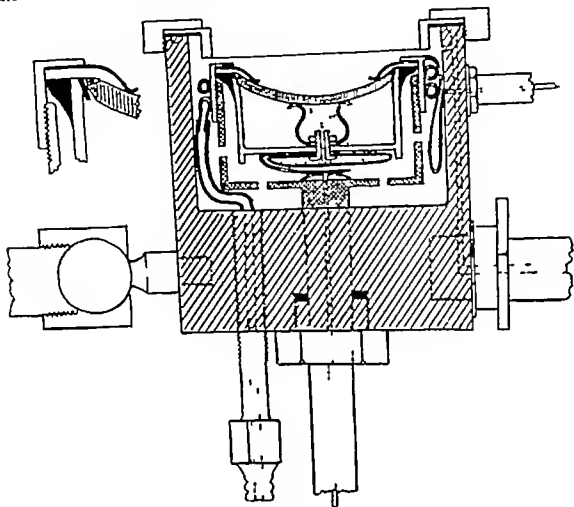


FIG 2 Diagram of the ultrasonic generator This is housed in a transparent bakelite container (diagonal lines) supported by a ball and socket joint (lower left) The container is sealed above by a cellophane diaphragm against which two small piezoelements are placed its interior is filled with transformer oil (clear) in which the quartz crystal vibrates The latter is mounted by its beveled edge on a black gasket (black) attached to the margins of a bakelite cup The interior of the cup forms an air filled chamber (stipple) behind the crystal Electrical leads (top and bottom center) connect with spring contacts on the two aluminum faces of the curved crystal The nodal suspension of the crystal by its beveled edge is shown in detail (upper left) Only one of the two water conduits (bottom left) is connected via neoprene tubing to the copper cooling tube on the outer side of the crystal collar A device for sucking out air bubbles under cell pressure is shown at lower right Neoprene gaskets, spreaders and air tubes are indicated in solid black

The entire generator is immersed in transformer oil contained in a transparent bakelite cylinder The oil is electrical insulating, for it is nonconducting Together they are efficient insulators against the passage of stray ultrasonic waves The oil transmits the focused beam of sound



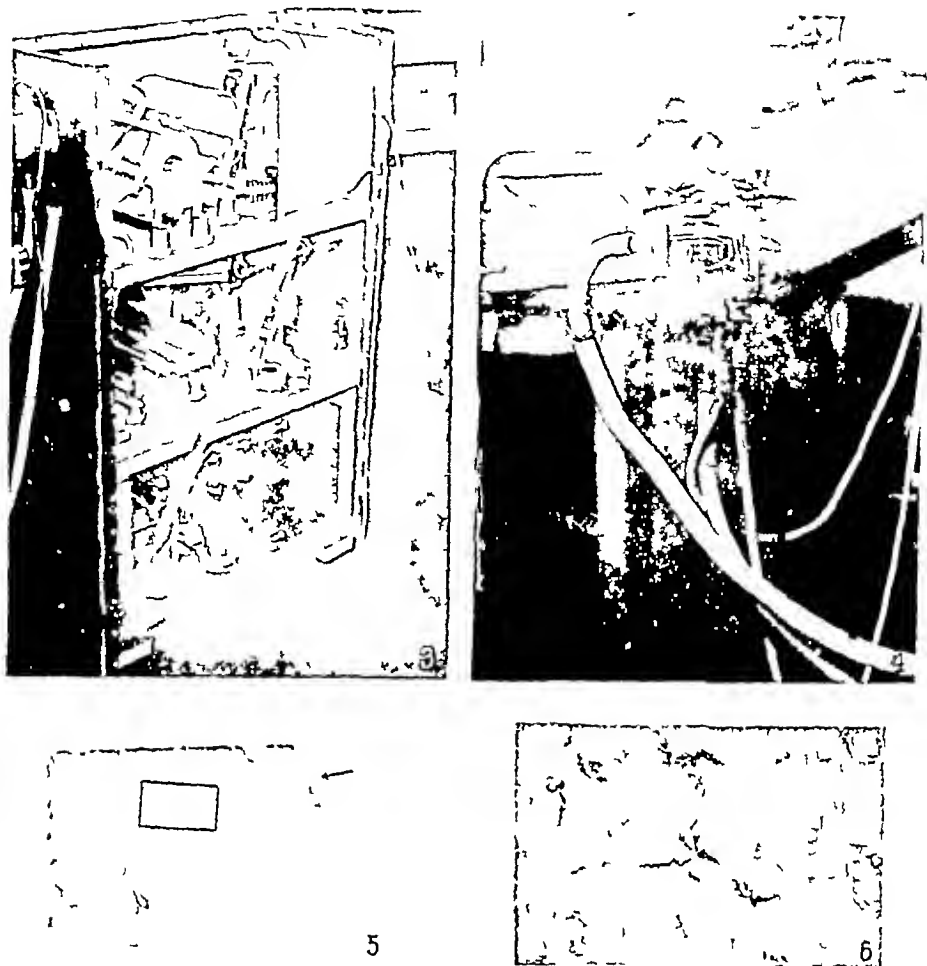


FIG 3 Photograph of the back of the radio-frequency generator showing the three stages on separate shelves See the diagram Fig 1 and text for details

FIG 4 The ultrasonic generator is supported by a ball and socket joint at the end of a 3 foot arm, and can be applied to the animal in any position The thick rubber tubing covers the electrical leads and the small tubes circulate cold water through the copper coil in the transformer oil surrounding the crystal (See Fig 2 for details)

FIG 5 Drawing of a thin section of liver showing region of application and focal point of ultrasound

FIG 6 Photomicrograph of a focal (left) and adjacent (right) region of the liver section in Fig 5

cellophane diaphragm which is in direct contact with the object or animal to be treated Fig 4 shows the entire set-up in actual operation with the head of an

anesthetized cat resting on the diaphragm 1.5 cm above the supersonic crystal. The bakelite cylinder is mounted on a universal joint at the end of a 3 foot movable arm, which extends from the side of the radio-frequency generator. This flexible mounting permits ultrasonic application in a wide range of positions and elevations.

### RESULTS

*A. Propulsion of Oil*—Both the intensity and form of the beam of focused ultrasound was made visible by floating several millimeters of oil on the surface of the cellophane diaphragm. When the current was turned on, the supersonic radiation pressure drove the oil upward from the center of the diaphragm in the form of a conical column, from the top of which oil droplets were thrown upward and outward so that the entire phenomenon resembled an erupting volcanic cone. The height of the cone proper is a reliable but crude indicator of the ultrasonic output. The oil cone height increases with the plate voltage.

TABLE I

*Strength of Electromotive Force and Amount of Ultrasonic Output as Measured by Height of Oil Cone—4 Minute Tests*

Test No	1	2	3	4	5	6
T 200 plate, volts	300	600	1600	1725	2025	2400
T 200 plate, amp	0.030	0.060	0.115	0.125	0.170	0.220
R.F. output, amp	0.100	0.200	0.520	0.700	0.800	0.900
Height of oil cone, cm	0.4	1.5	6.0	10.0	11.5	12.5

and the R.F. output. However, the height reached by the droplets thrown from the top of the cone is usually about twice that of the cone itself.







With the cooling system in constant operation, 4 minute runs were made at the power settings listed in Table I. At each setting, a constant ultrasonic output was obtained throughout the test period, as indicated by the constant maintenance of the height of the oil cone with constant meter readings. The specifications for the ultrasonic quartz plate used in the following tests, as well as in all subsequent experiments reported herein are round, diameter 5.08 cm, frequency 835 kc, curved to focus at a point 5.5 cm. from concave crystal surface, and 4 cm. above the center of the flat cellophane diaphragm.

It should be noted from Table I that with a plate current of 220 milliamperes and a potential of 2400 volts, there is a peak output of 900 milliamperes of R.F. current which produces a sufficient ultrasonic output to raise a solid oil cone 12.5 cm. high from the diaphragm with spray erupting to twice that distance. This measure of ultrasonic output for the one-half kw. of power with 2400 volts available can be compared for instance, with a maximum 10 cm. oil column attained from a flat quartz plate of equal diameter by Wood and

Loomis (1), who utilized 2 kw of power with 50,000 volts on the plate of the power tube

The chief factors contributing to the unusually high efficiency and ultrasonic output of this apparatus (with one-fourth the power and one-twentieth the voltage used by Wood and Loomis) are as follows (1) The master crystal oscillator control creates a stable and maximum power transfer from the R F to the ultrasonic crystal (2) The airchamber reflector behind the ultrasonic crystal at least doubles the output of ultrasound (3) The suspension of the crystal by cementing the nodal line at its edge to a flexible neoprene rubber gasket provides maximum freedom from dampening and stress during vibration (4) The focusing crystal drives the oil from the base towards the apex

TABLE II  
*Paraffin Melting Defects Produced by Focused Ultrasound*

Paraffin block	1	2	3	4	5	6
T-200 plate, volts	0 290	600	1600	1730	1730	1730
T-200 plate, amp	0 030	0 060	0 115	0 130	0 130	0 130
R.F output, amp	0 100	0 200	0 520	0 700	0 700	0 700
Total exposure time, sec	30	30	30	30	15	10
Time from 0 to power indicated, sec	5	5	5	5	5	1
Diagram of longitudinal section of melting defect						

of the radiation cone, where it accumulates and is piled up so as to exceed the 5.5 cm focal length of the crystal (5) The oil cooling system prevents the crystal temperature from rising sufficiently to lower its frequency, disturb its resonance, and so reduce the ultrasonic output

*B Paraffin Blocks*—Preservation and demonstration of the conical effects in solid form was obtained by resting cubes of paraffin (M.P. 58° C) in the film of transformer oil on top of the diaphragm just over the focus. A film of mineral oil, olive oil, lanolin, or normal salt solution must be between the diaphragm and the surface of the object irradiated to insure the displacement of all air. Air, if present, reflects and disperses the sonic beam. In the tests and experiments reported here we used 4 or 5 mm of transformer oil.






With the crystal 1.5 cm below the diaphragm, it was found by actual experiment that the focus, as indicated by the melting defect in the paraffin, was always 1.9 cm above the diaphragm. This means it was 3.4 cm above the

crystal, in contrast to the 5.5 cm. focal distance for which the crystal curvature was ground. The shorter focal length in actual practice can only be due to refraction effects which occur as the beam passes from the inside oil, through the diaphragm, outside oil film, and into the paraffin block.

Table II illustrates six representatives of several dozen experiments carried out with paraffin blocks at different intensities and exposure times.

Maximum focal with minimum base effects were obtained only with high intensity and short time ultrasonic exposures (blocks 5 and 6). The probable reasons for this phenomenon and its bearing on our basic problem will be taken up in the discussion.

TABLE III  
*Beef Liver Changes Produced by Focused Ultrasound*

Liver block experiment	1	2	3	4	5
T 200 plate, <i>volts</i>	600	1750	1750	2410	2410± +
T 200 plate, <i>amp</i>	0.060	0.120	0.120	0.220	0.220± +
R.F. output, <i>amp</i>	0.200	0.700	0.700	0.900	0.900±1.00 +
Total exposure time, <i>sec</i>	30	30	180	30	20
Time from 0 to power indicated, <i>sec</i>	10	10	10	10	Instantaneous
Diagram of longitudinal section of liver changes					

*C. Beef Liver Experiments*—Blocks of animal tissue were next placed on the film of oil on the diaphragm and irradiated in a manner similar to the paraffin blocks. Liver from a freshly killed animal was found to give the most easily observed results, because the strongly irradiated portions turned grayish brown, leaving the rest of the tissue a deep maroon color. Table III was compiled from a series of ten such experiments.

Focusing effects could only be obtained when full power was applied *instantaneously* as in liver block experiment No. 5 (Table III). See Figs. 5 and 6 for a microphotograph of a section through this liver block. A very small amount of cell destruction has occurred on the surface of the base. Lying above this, there is a region of unaffected liver cells, and finally a well defined focal point of severe destruction can be seen.

The significance of the foregoing findings on beef liver, and their bearing on the basic problem of achieving focal stimulation or destruction of cerebral tissue will be discussed in a later section.

*D Radiation of the Brain in Living Animals*—In view of the foregoing preliminary experiments with blocks of paraffin and liver tissue, it seemed probable that the present ultrasonic set-up with its high-frequency, limited R F power output, and small area of application to the scalp could not be expected to produce focal changes in the brain without simultaneous injury to the surface tissues, lying at the base of the cone of radiation. This proved to be the case, since all five animals (three cats and two dogs) showed more or less severe injury to the scalp in the area of application, while only those two animals which were exposed to radiations of maximum intensity showed any signs of local cerebral effects. In one dog, there was a transient injury to the upper portions of the precentral gyri, inferred from a weakness and incoordination of the hind extremities lasting about 16 hours. A cat, treated over the occipital visual area, showed blindness lasting for several hours which was followed by recovery.

#### DISCUSSION

An analysis of the results should give a better understanding of what occurs when a focused beam of ultrasound is applied to non-living and living material. For with a better comprehension of the processes involved, it should be possible to define more clearly the directions in which technical improvement in the generation and application of focused ultrasound must proceed if it is to be developed into a practical agent for the local modification of brain function.

The paraffin melting defects obtained and diagrammatically represented in Table II can be explained by simple thermodynamic principles which apply to the accumulation of heat ( $58^{\circ}\text{C}$ ) to produce local melting in a uniform medium such as paraffin. The chief determinants of the amount of local heat accumulated in unit of material for unit time are (1) rate of heat generation, and (2) rate of heat dissipation.

The rate of heat generation in any local region of the paraffin varies directly with the local intensity of ultrasonic radiation. The local intensity is dependent on the amplitude of crystal vibration which varies directly with the R F voltage applied to the quartz and with the concentrating effects of focusing. The latter increases from a minimum to a maximum per unit mass as one proceeds from the base to the focus of the radiation cone.

In contrast, the rate of local heat dissipation varies directly with the proximity of the heated region to cool non-radiated region of paraffin. Thus, at the focal spot, where the heat generation is greatest, its dissipation to immediately adjacent cool areas is also greatest, while a similar spot at the center of the base region, remote from any cool non-radiated paraffin has minimum dissipation.

It is this base-to-focus variation in the ratio of heat generation to heat dissipation which gives a base-to-focus gradient in heat accumulation, manifest in amounts of melting in these regions. The form of this base-to-focus gradient in heat accumulation, has been found in the paraffin block experiments to be reflected in the form of the melting defect. This form was found to vary with both the intensity and the duration of the supersonic output.

In paraffin block 1 (Table II) the intensity of the ultrasonic output was so low as to be insufficient to produce heat generation at the focus faster than it was conducted away by the cooler non-radiated adjacent regions. Hence, there are no focal signs of melting. In the base, however, despite the lower heat generation per equal unit of paraffin, the heat dissipation, especially near the center, is so slow that there is sufficient heat accumulation in 30 seconds to reach 58°C and to melt out the basal defect shown.

With an increase in intensity of ultrasonic output, the rate of heat generation may exceed the rate of its conduction from the focal region, so that there is sufficient accumulation of heat in 30 seconds to produce melting at the focus as well as at the base (see paraffin block 2, Table II).

With further stepping up of the intensity of irradiation, the rate of heat generation becomes still greater as compared to its rate of dissipation. Hence, the heat accumulation, as indicated by the melting defects at the focus and base, becomes proportionately larger and larger until they finally merge (paraffin blocks 3 and 4, Table II).

It is only when high ultrasonic power is applied for a short 15 or 10 second period that there appears a relatively greater heat accumulation at the focus with complete melting at this region, as compared to a centrally located incomplete melting at the base (blocks 5 and 6, Table II). This phenomenon is understandable when it is realized that the rate of heat generation is maximum the instant full power is applied and this maximum is maintained throughout, while the achievement of a maximum heat dissipation by conduction must be a sequel to its generation and takes more time. Therefore, when the exposure time is sufficiently short, heat dissipation becomes less and less a factor determining the amount of heat accumulated in a local area. Thus, when, in experiments 5 and 6 the exposure times are for 15 and 10 seconds respectively at high power, the amount of heat generated becomes the major determiner of the amount of heat accumulated at the base and focus respectively. This is in contrast with experiment 4 at the same power for 30 seconds, where a large melting defect occurred at the base.

The results of the beef liver experiments can now be analyzed. Fresh tissue is not a uniform medium like paraffin. Instead it consists of a multitude of protoplasmic interfaces which act to reflect, refract, and so to produce absorption of the ultrasonic radiations. Thus, the absorptive capacity of fresh beef

liver for supersound should be much higher than that of paraffin<sup>1</sup> This was found to be the case in the experiments in which the dosage was slowly increased to maximum intensities where only basal effects were produced (liver experiments 1 to 4, Table III) Apparently, the radiations were all absorbed before they reached the focus in sufficient intensity to produce any change in this region However, when full power was *instantaneously* applied, a well defined focus was obtained (experiment 5, Table III and Fig 5) Success under these conditions might be due to the transient but very high surge of power which always accompanies instantaneous switching-on of current This was registered on the R F ammeter as an initial reading of over 1 00 ampere, with an immediate dropping-back of the pointer to a stable maximum output level of 0 9 amperes Only when this initial peak surge of R F output occurred could sufficient ultrasonic intensity be obtained to produce a focal concentration strong enough to cause focal destruction in fresh liver tissue

The work of Dognon, Dognon, and Biancani (7), Harvey (8-10), and others shows that unicellular organisms are more vulnerable than multicellular organisms to ultrasonic waves Furthermore, with an increase in the size of the animal, its resistance increases, especially if it happens to acquire the protection of a shell covering

In the case of the experiments on living animals, there was ample evidence to indicate that with the intensities available, immediate tissue injury with irreversible changes resulting in scalp ulcerations occurred only when applications of at least 5 minutes were used at medium or high power Furthermore, the region of chief injury was in the superficial soft tissues at the base of the radiation cone where both heat conduction and blood circulation are less than at the focus in the brain The experiments of Gohr and Wedekind (11) emphasize the important rôle that circulating blood and tissue fluids play in protecting against the injurious effects of ultrasound applied *in vivo*, despite the high absorptive capacity of the tissues treated They exposed the abdominal skin of an anesthetized rabbit to supersonic waves There occurred a slight skin erythema with hyperemia, while inside there were peritoneal hemorrhages with dilation of the intestine However, when the same treatment was applied to an animal killed immediately before exposure, they observed rupture of skin vessels, changes in blood pigment, and innumerable perforations of the gut with passage of fecal matter into the peritoneal cavity The dissipation of heat should be especially effective in the brain, because of the richness of its vascular supply

A factor contributing to skin injury may have been the use of irritating trans-

<sup>1</sup>Dognon, Dognon and Biancani (7) found that liver tissue in a standard aluminum capsule had the same absorption as paraffin for ultrasound as indicated by temperature measurements However, the condition of the liver in regard to freshness and cell structure are not given

former oil rather than bland olive oil or normal salt solution as a skin contact medium. Frenzel (12), Pohlman, Richter, and Parow (13) have shown that ultrasound increases skin permeability, which, in conjunction with the radiation pressure, causes rapid absorption of drugs and other substances.

In the two animals which received exposures of maximum intensities over periods of 12 to 24 minutes respectively, behavior changes occurred which lasted for some hours, and which indicated damage in the local brain areas involved. However, this change was temporary. The refracting and reflecting effects of the layers of head tissue of different density such as skin, subcutaneous tissue, bone, membranes, and brain substance, must inevitably produce some diffusion of the focusing effects. When one of the animals was brought to autopsy just after recovery from the blindness produced, actual edema and hyperemia of the brain and pia were observed locally in the cortical visual area affected, while the remainder of the brain appeared normal.

#### *Future Developments and Applications of Focused Ultrasound*

As a result of this study, improvements can be made in the present focused ultrasonic generator which should enable one to produce local changes in tissues in general or in the brain in particular with a minimum of complicating injuries to superficial tissues. This basic problem has already been faced and partially solved in the case of x ray therapy. Here, the chief means of solving the difficulty have been by perforated lead screens, multiple beams, circular motion of the source of radiation, or by actual rotation of the object radiated so that the x ray beam sweeps over a wide area of skin surface. Because of the very short wave lengths involved, focusing has not been found practical with x rays. However, with the use of ultrasound, focusing is very practical in the frequencies lying above 250 000 per second, where the wave length in water is 6 mm. and less. The following lines of development would seem to be indicated in order to further reduce skin effects and increase focal changes.

- 1 Use of a non-irritating skin contact medium such as normal saline, olive oil, or lanolin on the diaphragm of the supersonic generator

- 2 Use of one-half to one-third of the present frequency. Since absorption increases as the square of the frequency in water (Langevin) and there is suggestive evidence pointing to almost equivalent absorption increase with frequency in tissues (Pohlman and Richter), this frequency reduction would seem advisable. With less absorption in the skin and superficial tissues, there would be a larger proportion of the radiation penetrating to the focus at any given output intensity.

- 3 Grinding of the crystal to a curve with as short a focal distance as is consistent with experimental needs. This obviously increases the intensity of the focal effect without increasing the skin effect.



4 Application of the treatment at maximum output intensity for the minimum period of time necessary to produce focal changes This follows from the results of the paraffin and liver block experiments, where it was found to be the most efficient means of applying ultrasound in order to obtain maximum deep focal with minimum superficial changes

5 Use of a mosaic of 4 to 6 two-inch curved crystals, all focusing on a common point This would not only increase the focal effect, but would spread the base effect over four to six times the skin area of the present set-up

6 Increase of the power of the R F generator to about 3 kw This should provide sufficient voltage to drive efficiently the mosaic of 4 to 6 two-inch crystals, each twice as thick (one-half the frequency) as the single quartz plate now in use

### CONCLUSIONS

1 An efficient generator of focused ultrasound has been designed, built, and successfully operated

2 The generator has been used to produce focal heating in the centers of paraffin blocks, and in a similar manner, focal areas of destruction were obtained deep in fresh liver tissue with minimal effects at the surface and no effects on the intervening tissue

3 In animals, focused ultrasound of high intensity produced local cerebral changes as inferred from behavior disabilities and as demonstrated at autopsy This local brain effect was achieved through intervening scalp, skull, and meninges The resulting behavior disabilities disappeared in from 2 to 16 hours

4 To date, it has not been possible to produce such brain changes without incidental injury to the skin and subcutaneous tissue lying at the base of the cone of radiation

5 Improvements in generation and application of the focused supersonic beam are suggested whereby it should be possible to increase still further the focal effects in the brain, with a corresponding decrease or elimination of complicating surface injury

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# THE EFFECT OF SPECIFIC POISONS UPON THE PHOTO-REDUCTION WITH HYDROGEN IN GREEN ALGAE\*

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In algae of the metabolic type represented mainly by *Scenedesmus*, it is possible to make the photochemical reduction of carbon dioxide proceed either with the evolution of oxygen or with the absorption of hydrogen (1). This offers new possibilities for analyzing the mechanism of photosynthesis. One of these is to compare the effects of specific poisons on photosynthesis with those on photoreduction, and the present paper contains contributions to this topic.

The selective poisoning of enzymes by substances such as cyanide, carbon monoxide, hydroxylamine, dinitrophenol etc., is a well recognized method for exploring complicated enzymatic systems *in vivo* and *in vitro*. This method has rendered and still renders valuable results in the studies of respiration and fermentation. Though successfully applied in his studies of photosynthesis by Warburg twenty years ago, selective poisoning did not continue to be of great use in elucidating the sequence of reactions in carbon dioxide reduction. The reason is that under the influence of poisons the normal process of photosynthesis always becomes inhibited in its entirety. No accumulation of intermediates nor any deviation in the chemistry of photosynthesis has been observed. The main result of all earlier investigations was that by diminishing the intensity of the radiation until it becomes the factor determining the rate of photosynthesis poisons which inhibit the "dark reactions" can be distinguished from those inhibiting the "light reactions" or both. It is only in recent years that comparison with the metabolism of purple bacteria (2, 3), the use of carbon isotopes (4), and the analysis with flashing light (5-7) have helped in assigning the effect of cyanide to a particular step in the course of the photosynthetic reaction.

The experiments reported below allow us to distinguish between the different parts of the mechanism of photosynthesis which are attacked either by cyanide, hydroxylamine, carbon monoxide, or dinitrophenol. Since these partial reac-

\* This is the first of three papers concerning the hydrogen metabolism of algae. In the text it will be referred to as Paper I, the two following ones as Papers II and III. The numbering of figures, tables, and literature citations is consecutive throughout the three papers in order to facilitate cross-references. The literature citations appear at the end of Paper III.

tions have to be arranged in a certain order to account for the observations, the experiments force upon us an extension of the existent theoretical picture of photosynthesis. Further it was found that the poisons act differently when introduced to the algal suspensions before instead of after adaptation to the hydrogen metabolism. The paper has been divided, therefore, into two parts, the first concerning the stationary state of photoreduction, the second the transitions from photosynthesis to photoreduction and back.

### Methods

The manometric measurement of normal photosynthesis following the well known procedures needs no comment. For the study of photoreduction, the algae (*Scenedesmus*, *Rhaphidium*, *Ankistrodesmus*) are suspended either in bicarbonate or phosphate buffers and incubated for several hours in atmospheres of either  $H_2$ ,  $H_2$  with 4 per cent  $CO_2$ ,  $N_2$ , or  $N_2$  with 4 per cent  $CO_2$ . Non-volatile poisons are stored in the side arm of the manometers, carbon monoxide was introduced into the vessel either pure or in a mixture with other gases. The only difficulty encountered was with cyanide solutions, because in protracted experiments the time is long enough to allow hydrocyanic acid to distill from the more concentrated solutions in the side arm into the algal suspension. It is necessary therefore to introduce the cyanide solution into the side arm of the vessel only a short time before use, either by letting the solution flow in through a vent stopper, or by smashing a glass bulb containing the poison by turning a ground-in paddle (see Fig. 14 in Paper III). The widespread image of a light source produced by a cylindrical lens was the simple means to illuminate a row of vessels with equal intensity, the light sources being incandescent lamps ranging from 200 to 1,000 watts. Several wire screens allowed for further adjustments of the intensity. It should be pointed out that photoreduction in unpoisoned cells tolerates only rather low intensities lying within a range, where in normal photosynthesis the rate would be proportional to the intensity.

### Part I Photoreduction in the Presence of Poisons

(a) *Cyanide*—It is well known that respiration as well as photosynthesis in plants is influenced by cyanide. Usually it is possible to separate these reactions from each other by means of the proper concentrations of poison because the sensitivity towards cyanide is different. With rising concentrations of cyanide either photosynthesis (e.g. *Chlorella* (8)) or respiration (e.g. *Scenedesmus* (9)) is poisoned first, leaving the other process more or less undisturbed and thus revealing whether or not any connection exists between these metabolic processes.

While the nature of the cyanide inhibition appears to be similar in many plants, the amount of cyanide necessary to attain the same degree of inhibition varies not only from species to species but also with time in the same plant (Goddard (10)). The simplest explanation is that a plant cell containing a large surplus of the respective enzyme molecules will be less sensitive to the poison than a cell containing only a barely sufficient number. Putting

half the number of enzyme molecules out of action may mean only a slight inhibition of the overall reaction in the first case and 50 per cent inhibition in the second. It is therefore more or less a matter of chance whether in a certain plant two different reactions sensitive to cyanide will be inhibited at sufficiently different concentrations of the poison so that it is possible to separate and to distinguish the effects.

In *Scenedesmus* there are at least three cyanide-sensitive reactions, 1), respiration, 2) photosynthesis and photoreduction, and 3) the adaptation

TABLE I  
*Effect of Cyanide on Photoreduction*

0.06 cc. of cells of *Scenedesmus D<sub>1</sub>* in 4 cc. of 0.025 M bicarbonate. Gas phase H<sub>2</sub> 4 per cent CO<sub>2</sub>. Temperature 25°. Preceding anaerobic dark period 12 hours.

Condition	Time min	1	2	3
		Rates of pressure changes in $\frac{\text{mm}}{10 \text{ min}}$		
Light. 450 lux	20	-16	-17	-18
Dark	60	-0.5	-0.5	-0.5
Dark, HCN added	10	—	$1.25 \times 10^{-4}$ M HCN	$1.25 \times 10^{-3}$ M HCN
Light. 450 lux	15 (15)*	-17	-9	-2
940 lux	5 (20)	-32	-12	-2
4750 lux	10 (30)	-94	-40	-5
4750 lux	5 (35)	-86 (turn)	$\pm 0$	$\pm 0$
940 lux	10 (45)	-40	+5	+4

\* Numbers in parentheses indicate total time elapsed since the beginning of illumination

to photoreduction. It is easy to distinguish between the sensitivity to cyanide of photoreduction and of the adaptation reaction because the same concentration of cyanide produces quite different results when added aerobically before the adaptation or anaerobically after its completion. Cyanide, however, has also a tendency to enhance the return to aerobic conditions. This fact makes it difficult to decide whether photoreduction, which is clearly less sensitive than the adaptation reaction, is on the other hand more sensitive to cyanide than normal photosynthesis. In Table I the algae have been under anaerobic conditions for a period of many hours before cyanide was added. The inhibition of photoreduction caused by  $1.25 \times 10^{-4}$  M cyanide is much greater than of photosynthesis. Especially at low light intensities this concentration of cyanide hardly diminishes the rate of photosynthesis in *Scenedes*

*mus* (see Table I in reference 9) Are we allowed to conclude that the actual photochemical reduction of carbon dioxide is more sensitive to cyanide when proceeding with the absorption of hydrogen than with the production of oxygen? The data in Table II indicate that under anaerobic conditions the effect of cyanide must be a complex phenomenon and not due merely to an inhibition of the reduction of carbon dioxide. The presence of hydroxylamine in addition to cyanide increases the rate of carbon dioxide reduction as compared with the low rate found in presence of cyanide alone. We shall discuss

TABLE II

*Inhibition of Cyanide Inhibition by Hydroxylamine*

0.037 cc of cells of *Scenedesmus D<sub>1</sub>* in 4 cc of 0.025 M bicarbonate. Temperature 26°. Gas phase air, 6 per cent CO<sub>2</sub> and H<sub>2</sub>, 4 per cent CO<sub>2</sub>. Preceding dark period 11 hours. Hydroxylamine added 55 minutes before experiment, cyanide added 25 minutes before. Light intensity 500 lux.

Concentration of poisons	Air/CO <sub>2</sub> +c.mm O <sub>2</sub>		H <sub>2</sub> C/O <sub>2</sub> -c.mm H <sub>2</sub>			
	—	2 × 10 <sup>-4</sup> M HCN	—	2 × 10 <sup>-4</sup> M HCN	1 × 10 <sup>-3</sup> M NH <sub>2</sub> OH	1 × 10 <sup>-3</sup> M NH <sub>2</sub> OH 2 × 10 <sup>-4</sup> M HCN
Rate of gas exchange in c.mm /5 min during 10 min, following 30 min of continuous illumination	+6.5	+5.5	-13	-2.1	-5.2	-4.0

(Experiment continued in Fig. 7)

this observation more in detail later in connection with the reactions leading to the return to normal aerobic photosynthesis. One thing, however, appears to be certain—normal photosynthesis is not more sensitive to cyanide than photoreduction. This is of some importance because it has been stated before (1) that the difference between the two forms of carbon dioxide reduction consists probably only in the ways by which the oxidized photoproducts are removed. They are either decomposed with liberation of free oxygen or reduced to water by hydrogen. If this be true our results with cyanide would prove that it is not the oxygen-liberating system which is responsible for the well known cyanide inhibitions of photosynthesis. Since this agrees with the observation on purple bacteria (2), as well as with those on the dark

absorption of carbon dioxide in plants (Ruhen, S, *et al* (4)) and the flashing light experiments of Weller and Franck (6), we should return to Warburg's (8) very first conception and say that it is the fixation of carbon dioxide to the assimilatory system preceding all the photochemical reactions which is predominantly inhibited by cyanide in normal photosynthesis

(b) *Hydroxylamine*— $\text{NH}_2\text{OH}$  is a poison which in very low concentrations inhibits photosynthesis strongly in all plants so far tested (11) Since hydroxylamine is a specific poison for catalase, this similarity was considered, until 5 years ago, as being in favor of the assumption that it is indeed catalase which takes part in photosynthesis by splitting hydrogen peroxide formed photochemically. It was easy to disprove this hypothesis by separating catalase activity and photosynthesis in cyanide poisoned cells and by studying the action of dilute hydrogen peroxide solutions upon the photosynthetic mechanism (9) In addition Emerson (12) had found that variations in the ability to split  $\text{H}_2\text{O}_2$  were independent of the rate of photosynthesis in different cultures of *Chlorella*. Nevertheless the similarity which exists between oxygen production in illuminated cells and catalase activity indicates that the oxygen liberating system, though not identical with catalase, might be an enzyme of similar constitution and properties (13)

The experiment in Table III shows the relative indifference of the photo-reduction with hydrogen towards hydroxylamine in concentrations several times greater than necessary for complete inhibition of photosynthesis. Hence it follows that hydroxylamine in small concentrations is a specific poison for the oxygen liberating system only. Further proof is given in the experiments of Fig. 1 and Table XXV in Paper III.

This clear difference between the effect of small amounts of cyanide and of hydroxylamine on photoreduction prompted an experiment by Weller and Franck (6) as to the dependency of the hydroxylamine inhibition on the light intensity. Warburg (8) showed long ago that cyanide has no effect on photosynthesis at intensities where the rate of the photochemical reactions determines the overall rate of the process. Contrary to the cyanide effect the inhibition of photosynthesis in *Chlorella* by hydroxylamine was found to be as great at low as at high intensities. Since the liberation of oxygen from the intermediary oxidized substances ("peroxides") should be a typical dark reaction similar to the fixation of free carbon dioxide in the partial reaction initiating the reduction, this result is hard to understand.

Following Gaffron's hypothesis (14) that the induction period in photosynthesis is due to the reversible inactivation of some part in the oxygen liberating enzyme system, Franck, French and Puck (15) concluded that the amount of active enzyme present must be proportional to the rate of photosynthesis. Using the same argument Weller and Franck explained their results by suggesting that, if the amount of active enzyme remains propor



tional to the rate of photosynthesis, hydroxylamine could inhibit always the same percentage of the total flow of photosynthesis

As such, this explanation is satisfactory Yet we must be prepared for further complications, because increasing the concentration of hydroxylamine

TABLE III

*Effect of Hydroxylamine upon the rates of Photosynthesis and of Photoreduction*

0.025 cc of cells of *Scenedesmus* in 4 cc of 0.05 M bicarbonate Gas phase (a) air, 4 per cent CO<sub>2</sub> (b) H<sub>2</sub>, 4 per cent CO<sub>2</sub> Temperature 25° pH 7.5

No of experiment		1	2	3	4	5
Volume of vessels, cc		15.4	14.9	15.2	15.0	15.4
	Observation time	Rate of gas exchange in $\frac{\text{mm}}{10 \text{ min}}$				
(a) Air CO <sub>2</sub>						
Dark	15 min	—	—	$+\frac{M}{10,000} \text{NH}_2\text{OH}$	—	$+\frac{M}{5000} \text{NH}_2\text{OH}$
Dark	10 min	+1	-1	0	-1	0
Light 2200 lux	5 min	+10	+10	+4	+10	±0
Light 2200 lux	10 min	+11	+11	+6	+11	+1
Dark	5 min	±0	±0	±0	±0	±0
(b) Hydrogen, CO <sub>2</sub>						
Dark	16 hrs	-4	-4	-2	-4	-0.7
Light 1100 lux	15 min	-20	-22	-22	-20	-2
Dark	20 min	—	$+\frac{M}{1000} \text{NH}_2\text{OH}$	—	$+\frac{M}{5000} \text{NH}_2\text{OH}$	—
Light 1100 lux	5 min *	-26	-12	-22	-26	-4
Light 1100 lux	5 min *	-45	-27	-40	-42	-4
Dark	6 hrs	At the end of this period 2.5 per cent of O <sub>2</sub> added				
Dark, after turn to aerobic conditions	20 min	-3.5	-3	-3	-4	-4
Light 2200 lux	40 min	+10	±0 → +3	+8	+1.5	+6

\* Sudden increase in rate Compare Table XXV, Paper III

in the preceding experiments beyond that for practically complete inhibition of normal photosynthesis leads to an inhibition also of photoreduction. Photoreduction is only relatively, not absolutely, insensitive towards hydroxylamine. This is made clear by the data given in Tables II, III, IX, and XXV (in Paper III) and Fig. 1. Rather large concentrations of this poison ( $\frac{M}{800}$  to  $\frac{M}{30}$ ) (added after adaptation, as required throughout this set of experiments) quickly diminish the rate of hydrogen uptake to about one-half

Afterwards the cells, left in contact with say  $\frac{M}{100}$  to  $\frac{M}{800}$  of this poison for a period of a day, will display rather irregular responses. Eventually the inhibition may become more and more pronounced, even total, or the effect of the poison may fade slowly away. Such side reactions may be expected, because hydroxylamine can react with all sorts of aldehyde and keto groups. The disappearance of the inhibiting effect with time is markedly accelerated in the light. This has been observed already by van Niel (personal communica

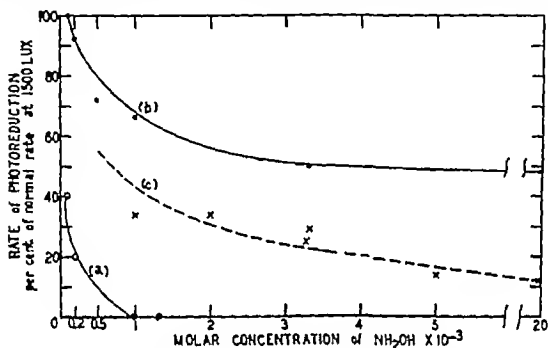


FIG. 1 Inhibition of the rate of photoreduction in *Scenedesmus* as a function of the hydroxylamine concentration. •—• percentage inhibition found within 1 hour after addition of the poison. x—x, inhibition 10 to 12 hours later. —○— effect of hydroxylamine on photoreduction when added aerobically before the algae are adapted to the hydrogen metabolism.

tion) Leaving aside such irregularities, we find according to Fig. 1 that the maximum inhibition achieved during the first hour or two is not more than 50 per cent to 70 per cent even with very high concentrations of hydroxylamine.

This is a strange feature, which appears even more complicated if we consider the influence of the light intensities upon the extent of the inhibition. Fig. 2 shows that doubling the intensity of irradiation may cause a sudden drop in the rate of photoreduction in strongly poisoned algae while Table IX and Fig. 7 in Part II demonstrate the more common case where the rate in poisoned algae still rises with the intensities. The persistence of ca 50 per cent of the initial rate of photoreduction after poisoning with excessive amounts of hydroxylamine has its counterpart in a few observations where algae apparently

normal and unpoisoned doubled their initial rate of photoreduction at a given low light intensity without any visible reason (See Table III above and Table XXV in Paper III)

(c) *Effect of Dinitrophenol on Normal Respiration and Photosynthesis in Chlorella*—There is no doubt that dinitrophenol acts in quite another way upon enzymatic reactions than the poisons discussed above (16). The literature on the catalytic or anticatalytic effects of substituted phenols in living cells has increased rapidly during recent years and it is impossible to cite all the relevant papers. As far as I can see, there are no reports, however, about the influence of dinitrophenol on photosynthesis in plants. Before discussing

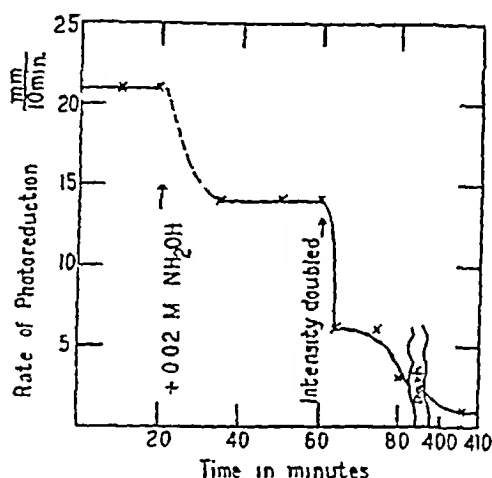


FIG. 2 Increase of the inhibition of photoreduction caused by excessive amounts of hydroxylamine under the influence of a higher light intensity

the influence on photoreduction, we have to consider the effect on normal photosynthesis

Table IV gives an instance with *Chlorella pyrenoidosa*, which is the "standard" test object in this field. We shall consider first respiration. All the observations made by Krahle and Clowes (17) on the respiration of the sea urchin egg are easily confirmed with *Chlorella*. Very small concentrations of dinitrophenol have a stimulating effect. With increasing concentration the stimulation, after reaching an optimum, turns into an inhibition which eventually surpasses 90 per cent (fermentation starting). The prominent part played by the hydrogen ion concentration in connection with the effect of dinitrophenol is reflected by the complete recovery from the total inhibition of respiration (and photosynthesis) after changing the pH in the medium from 5 to 6.5

A true stimulation of photosynthesis was not observed and is not to be expected. Photosynthesis is not affected by concentrations of dinitrophenol which stimulate respiration. This observation agrees with earlier ones on

the differentiating effect of cyanide indicating that the normal tissue respiration in algae has no connection with the photochemical system (13). At the proper pH very small concentrations of dinitrophenol inhibit photosynthesis strongly. The inhibition can be made to disappear by making the suspension medium more alkaline just as easily as in the case of the inhibited respiration (Table IV). After prolonged treatment with the poison, particularly under anaerobic conditions, the reversibility is lost and the cells appear permanently damaged.

TABLE IV

*Effect of Dinitrophenol on the Metabolism of the Alga Chlorella pyrenoidosa*

Comparison of stimulating and inhibiting doses at two different hydrogen ion concentrations. 0.025 cc. of cells in 6 cc. of phosphate buffer. Temperature 25°. Gas phase air-4 per cent CO<sub>2</sub>. Normal rate of respiration = 10. Normal rate of photosynthesis = 100.

Concentration of dinitrophenol.		$3.3 \times 10^{-4} M$	$1.6 \times 10^{-4} M$	$0.8 \times 10^{-4} M$
	pH			
Respiration (dark 40 min.)	5.0	18	2	0
	6.2	10	11	19
Photosynthesis (after 15 min. at 900 lux)	5.0	100	10	3
	6.2	100	100	100
Respiration (dark 30 min.)	5.0	12	0	10*
	6.2	7	6	13
Photosynthesis (35 min. at 900 lux)	5.0	105	10	110*
	6.2	100	110	100
(10 min. at 1700 lux)	5.0	170	6	120*
	6.2	150	150	150

\* Here the pH has been changed from pH 5 to pH 6.5 the metabolic rates recovered from the inhibition.

(d) *Effect of 2,4-Dinitrophenol upon Photoreduction in Scenedesmus and Ankistrodesmus*—All that has been observed concerning the effect of dinitrophenol upon normal photosynthesis in *Chlorella* pertains also to those algae which have a hydrogenase system. Some unimportant differences are found as to the relative sensitivity of respiration and photosynthesis towards the same concentration of poison.

Turning to photoreduction we find in Table V data which show that the anaerobic process is susceptible to the poison to the same extent as photosynthesis. Hence the reactions concerning the evolution of oxygen are not involved in this particular inhibiting effect, at least not manifestly so. In all probability it is the transfer of hydrogen to carbon dioxide which is inhibited.

(Compare references 16 and 17, Tables XVII to XIX in Paper II, and Table XXVI in Paper III) The same results were obtained with the alga *Ankistrodesmus*

Testing the inhibiting power of different concentrations of dinitrophenol (Fig 3) an unusual induction period was observed on the 2nd day of an experiment for which a satisfactory explanation cannot be offered at present

TABLE V

*Dinitrophenol Inhibition of the Gas Exchange in Scenedesmus*

0.027 cc of cells in 4 cc of 0.05 M phosphate buffer pH 6.0 Temperature 25°

Volume of vessel, cc		1	2	3
Final concentrations of dinitrophenol		15.2	15.0	15.4
		—	$2 \times 10^{-4}$ M	$2 \times 10^{-4}$ M
	Observation time	Rates of gas exchange in $\frac{\text{mm}}{10 \text{ min}}$		
(a) Air, 4 per cent CO <sub>2</sub>				
Dark Respiration	40 min	-0.8	-2.0	-0.2
Light 2000 lux Photosynthesis (corrected for respiration)	20 min	+17	+15	+2
Dark (not measured)	12 hrs	—	—	—
Dark Respiration	30 min	-0.5	-1.5	+0.5
Light 2000 lux Photosynthesis	35 min	+11	+7	±0
(b) H <sub>2</sub> , 4 per cent CO <sub>2</sub>				
Dark Adaptation	6 hrs	-1.0	-0.6	-0.2
Light 550 lux Photoreduction	15 min	-38	-22	±0
Temperature increased to 38°				
Light 550 lux Photoreduction	15 min	-38	-27	-2

(c) *Carbon Monoxide*—Carbon monoxide, as shown in Table VI, interferes very strongly with photoreduction

The finding that photoreduction is so sensitive towards carbon monoxide at last gives a fully satisfactory explanation for experiments published several years ago, before the photoreduction with hydrogen in algae had been discovered. It had been a general experience among students in this field that carbon monoxide has no influence upon photosynthesis. The only effect ob-

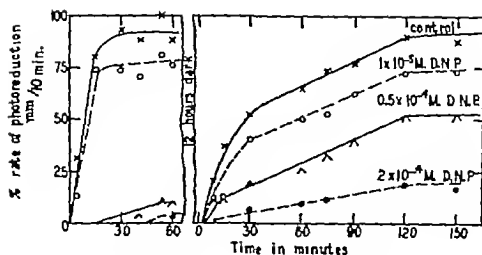


FIG 3 Inhibition of photoreduction by 2,4-dinitrophenol. *Scenedesmus* suspended in  $M/30$   $KH_2PO_4$  pH ca 5.0

TABLE VI

*Effect of Carbon Monoxide on Photoreduction*

0.03 cc. of cells of *Scenedesmus*  $D_1$  in 4 cc. phosphate buffer pH 6.7 (retention of  $CO_2$ )  
Temperature  $25^\circ$  Gas phase initially  $H_2$ , 4 per cent  $CO_2$

	Time	Rate of gas exchange in $\frac{\text{mm. pressure change}}{10 \text{ min}}$	
		1	2
(a) $H_2$ 2 per cent $CO_2$ Dark	200	-1.4	-1.2
(b) Dark new gas	60	78 per cent $CO$ 2 per cent $CO_2$ 20 per cent $H_2$	78 per cent $N_2$ 2 per cent $CO_2$ 20 per cent $H_2$
Light	5	$\pm 0$	-14
1000 lux	(10) 5	+2	-16
	(13) 3	+3	-7
	(15) 2	+5	0
	(25) 10	+6	+5
Dark	15	-1	-1
	110	0	-2

\* Numbers indicate total time elapsed since beginning of illumination.

served had been an inhibition of respiration in certain plants. In 1935 it was found (18) that after an anaerobic incubation period photosynthesis started quite differently when the algae had been kept in nitrogen than when they had been in carbon monoxide, prior to illumination. At that time it was

assumed that photosynthesis was inhibited initially by products of fermentation (see also (19)) which became oxidized as soon as free oxygen was liberated. Carbon monoxide was supposed to inhibit this secondary utilization of oxygen and thereby, indirectly, the recovery of photosynthesis.

In 1940, it was realized that the gas exchange during the "anaerobic induction period" is due to an internal photoreduction of carbon dioxide without the formation of free oxygen (20). Now we see that it is this reaction (involving in *Scenedesmus* the liberation (Paper II) and absorption of molecular hydrogen) which is sensitive to carbon monoxide. A similar effect of carbon monoxide on photoreduction had been observed previously with the photochemically active red sulfur bacteria (Thiorhodaceae) (21).

## *Part II Transition Reactions*

### *1 Adaptation to Photoreduction*

(a) *General Observations*—In addition to what has been published about how the "reduced state" of the photochemical mechanism is established in algae, the following observations ought to be mentioned. At room temperature, it takes about 2 hours of anaerobic incubation in the presence of hydrogen for *Scenedesmus* to display photoreduction upon irradiation. (At 36° this "adaptation" process is considerably shortened.) Yet algae in contact with hydrogen only for the minimal periods are likely to return to normal photosynthesis at much lower intensities than those which were allowed to ferment in the dark for a long period of time. The reversion threshold of the tolerable intensity rises at first with the length of the preceding anaerobic dark period. Part of the adaptation occurs also in an atmosphere of nitrogen, but there is a difference between the anaerobic metabolism in nitrogen and in hydrogen, as shown in Table XIa and XIb in Paper II. Under anaerobic conditions the algae ferment, forming organic acids and free carbon dioxide. The amount of acids and carbon dioxide is approximately the same whether fermentation takes place in nitrogen or in hydrogen. The difference consists in the metabolic exchange of hydrogen. In hydrogen, the algae absorb varying amounts of hydrogen (compare reference 1) while in nitrogen hydrogen is liberated (compare Table XI in Paper II). The rate of hydrogen uptake in the dark declines rapidly with time. With sensitive instruments, however, one can show that this uptake persists as long as the algae are kept under anaerobic conditions and continue to ferment. (Experiments with differential manometer Rieke, unpublished data.) Part of the hydrogen absorption during the first few hours appears to be necessary for a successful adaptation to photoreduction.

(b) *Cyanide Inhibition of Adaptation*—In *Scenedesmus* (several strains) *Rhaphidium*, *Ankistrodesmus*, the adaptation to the hydrogen metabolism is readily inhibited by small concentrations of cyanide. Of all metabolic processes

the adaptation reaction appears to be the one most sensitive to cyanide poisoning. Complete failure to attain the "reduced state" of the photochemical mechanism can often be observed with a concentration of cyanide which

TABLE VII

*Cyanide Inhibition of Adaptation to Photoreduction*

0.017 cc. of cells of *Rhaphidium polymorphum* in 0.033 M phosphate buffer pH 5.3  
 Temperature 25 Gas phase air, air/4 per cent CO<sub>2</sub> H<sub>2</sub>/4 per cent CO<sub>2</sub>

No.	1	2	3
Volume of vessels cc.	14.9	15.4	16.0
Volume of liquid cc.	5.0	3.2	3.2
Side arm	—	0.2 cc. 6 per cent NaOH	0.2 cc. 6 per cent NaOH
Gas phase	Air; CO <sub>2</sub>	Air	Air
Concentration of poison	—	—	+ 10 <sup>-4</sup> M HCN
(a) Respiration			
Dark, 75 min	—4	—11	—7
	—12.3 c.mm. O <sub>2</sub> + 12.0 c.mm. CO <sub>2</sub>		—8.3 c.mm. O <sub>2</sub>
No.	4	5	6
Concentration of poison	—	+10 <sup>-4</sup> M HCN	+10 <sup>-4</sup> M HCN
Volume of vessels, cc.	15.3	15.0	15.4
Volume of liquid cc.	4.0	4.04	4.4
Rate of pressure changes in $\frac{\text{mm.}}{10 \text{ min.}}$			
(b) Light, 4000 lux. Photosynthesis			
1) Gas phase air/CO <sub>2</sub>	+24	+20	+10
2) Gas phase H <sub>2</sub> /CO <sub>2</sub> , trace O <sub>2</sub>	+26	+20	+8
(c) Adaptation. Dark 14 hrs.			
Gas phase H <sub>2</sub> /CO <sub>2</sub>	—2	—0.25	+0.04
(d) Light 2000 lux Photoreduction			
Gas phase H <sub>2</sub> /CO <sub>2</sub>	—79	+3	+2

scarcely inhibits respiration, fermentation, photosynthesis, and photoreduction, once the latter has been established. Compare the data of Table VII with those of Tables I and II.

When no adaptation occurs the algae absorb either no hydrogen during the anaerobic incubation period or only a fraction of the amount taken up by the unpoisoned control suspension. This indicates that the reduction of some



substance (catalyst or enzyme) is essential for the photochemical utilization of hydrogen donors in place of oxygen liberation, and that, once reduced, the rôle of the reduced enzyme in photoreduction (if any) is influenced much less by cyanide. In this connection it should be noted that in the presence of cyanide the system is extremely sensitive to oxygen. Small amounts of oxygen otherwise made harmless by reduction cause the irreversible return to aerobic photosynthesis (see Table XXIII in Paper III).

(c) *Effect of Hydroxylamine on Adaptation*—In general, hydroxylamine is a much more potent poison for photosynthesis than cyanide. With concentrations as low as  $10^{-4}$  M the inhibition can be almost complete. We found that the adaptation reaction also is sensitive towards hydroxylamine, but not more so than photosynthesis. Both reactions are inhibited to about the same extent. Complete inhibition of photosynthesis is accompanied by complete inability of the cells to adapt themselves to photoreduction. Partial inhibition of photosynthesis corresponds to a partial inhibition of the adaptation reaction. We observe that less hydrogen is absorbed by the algae during the incubation time and that upon illumination photoreduction begins and continues at only a fraction of the rate found in the unpoisoned algae. Here again, as in the case with cyanide, it is striking to see that the same amount of poison added after full adaptation does not influence photoreduction at all (Table III above and Table XXV in Paper III).

Different samples of algae react somewhat differently towards the same concentration of poison. From the many data, however, emerges again the fact that with increasing amounts of poison the degree of inhibition increases in two big steps rather than gradually. The amount of poison is either not sufficient to inhibit much of the adaptation reaction and the subsequent rate of photoreduction is hardly diminished, or the adaptation reaction is conspicuously reduced and the corresponding rate of photoreduction is only about one-half of that of the control, or the inhibition of the adaptation is complete and neither photoreduction nor photosynthesis is observed in the light. The individual variations from this scheme are large, but its general validity cannot be overlooked.

(d) *Dinitrophenol Inhibition of Adaptation*—Since dinitrophenol inhibits photosynthesis and photoreduction to the same degree, little difference, if any, is observed between adding the poison before or adding it after adaptation. The adaptation reaction seems to be affected least of all. However, not enough experiments have been done to clear up the complication arising from the fact that dinitrophenol is a strong inhibitor for the fermentative production of hydrogen in the dark (Paper II).

(e) *Inhibition of Adaptation by Carbon Monoxide*—In the presence of carbon monoxide instead of nitrogen, the reduction reactions enabling the algae to utilize hydrogen are inhibited. In Fig 13 (Paper II) we see that algae incubated in these two gases differ afterwards in their reaction with hydrogen.

## 2 *Transition from Photoreduction to Photosynthesis under the Influence of Light*

(a) *General Observations*—Algae adapted to photoreduction either with free hydrogen or internal hydrogen donors will invariably return to normal photosynthesis if the intensity of radiation surpasses a certain threshold value (which varies with internal factors of the particular strain of algae). The speed of this "turnback" reaction is a function of the excess intensities. It is easier to understand the meaning of the experiments described below in the terms of the explanation we have presented for this effect of intense light (1, 22). We shall repeat it therefore. The explanation was that the intermediate oxidized products resulting from the photochemical dehydrogenation of water, when formed at a rate higher than that of their reduction by the hydrogen donors, start to reoxidize that part of the mechanism which had to be reduced during the adaptation time in the dark. In the course of this process the hydrogenase system becomes inactivated. Once this has been completed, the oxidized photoproducts will again be decomposed with the liberation of oxygen. This view is supported by the following experiments. Low partial pressures of hydrogen speed up the turnback while low pressures of carbon dioxide retard or prevent it (Fig 4 and Table VIII). The efficiency of the truly photochemical process in a plant can be tested by illumination in flashing light. The dark intervals between the flashes are made so long that all dark reactions initiated by the absorption of light run to completion before the next flash appears. Under these circumstances the highest yield per flash obtained by increasing the intensity of the flashes is called the flash saturation value, a characteristic of the photochemical system. Rieke (7) found that the value for the saturation of photoreduction in flashing light is the same as in photosynthesis. (Compare Emerson and Arnold (5).)

One sees further in Fig 4 that a low partial pressure of hydrogen supports photoreduction nearly as well as a high one during the first moments of illumination. We believe therefore that the adsorption of hydrogen to the hydrogenase not the rate of the reduction of the intermediate oxidized products is a function of the hydrogen partial pressure.

(b) *Turnback in Presence of Cyanide*—We have seen above that cyanide in very small concentrations prohibits the adaptation; we may say it prevents the reduction of an enzyme and fixes it in its oxidized state. It can hardly be doubted that the turnback is literally the reversal of the adaptation reaction and includes the reoxidation of some catalyst. In fact when an illumination with a high intensity has lasted only a short while, the "damage" done to the reducing system is repaired in a short time, provided the turnback has not been complete (Table I). That means, as we believe, that the remaining active part of the hydrogen transferring system aids in the reactivation of the oxidized part. In presence of cyanide that is not the case. No recovery is possible because all enzyme molecules which happen to be oxidized will remain so. (See Table XXIV in Paper III.) The effect of cyanide in

general can be summarized by saying that it speeds up the turnback once it has started

TABLE VIII

*Dependence of the Inactivation by Light on the Presence of Carbon Dioxide*

0.04 cc of cells of *Scenedesmus obliquus* in 4 cc of  $1.25 \times 10^{-4}M$   $KH_2PO_4$  and culture medium Temperature  $25^\circ$  Several hours adaptation prior to the experiment

	Time	(a) H <sub>2</sub> with 8 per cent CO <sub>2</sub>	(b) H <sub>2</sub> (CO <sub>2</sub> absorbed by KOH)
		Rates of pressure changes in $\frac{mm}{10\text{ min}}$	
2000 lux Photoreduction and turnback in light	After 15 min	-18	$\pm 0$
	After 50 min	+4	$\pm 0$
Dark Oxyhydrogen reaction (compare Paper III)	50 mm of O <sub>2</sub> introduced into each vessel		
	15 min	-1	-12
	Total pressure changes due to 50 mm of O <sub>2</sub>		
	300 min	-19	-122

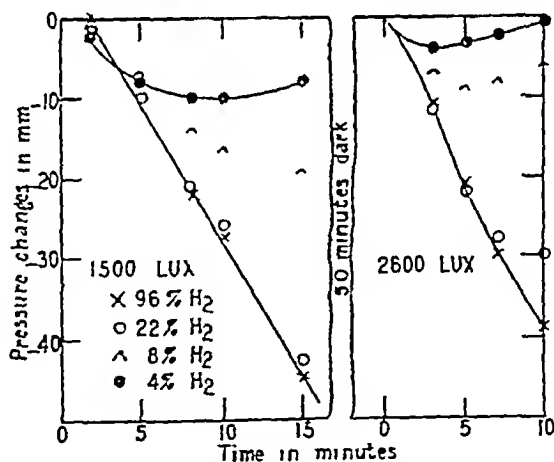


FIG 4 The time course of photoreduction as a function of the partial pressure of hydrogen *Scenedesmus D<sub>3</sub>* in culture medium Preceding adaptation during 16 hours in H<sub>2</sub>, 4 per cent CO<sub>2</sub> All mixtures of hydrogen and nitrogen used in this experiment contained 4 per cent CO<sub>2</sub>

(c) *Turnback in Presence of Hydroxylamine*—In small concentrations, hydroxylamine is a strong poison for photosynthesis and hardly an inhibitor for photoreduction (see Table III) Consequently, the “photoperoxides” should accumulate under the influence of excessive light exactly as in unpoisoned cells and oxidize and inactivate the hydrogenase system It was

found, however, that hydroxylamine interferes with the turnback reaction. Its effect is opposite to that of cyanide. It is retarding instead of enhancing. In other words, the mechanism of photoreduction is protected against the turnback by the presence of hydroxylamine. Fig 5 shows how the algae adapted to photoreduction when irradiated with an intensity surpassing the threshold intensity will return to photosynthesis in the course of a few minutes, while the presence of some hydroxylamine enables them to continue with photoreduction. The units of the ordinate in this figure are actual manometer

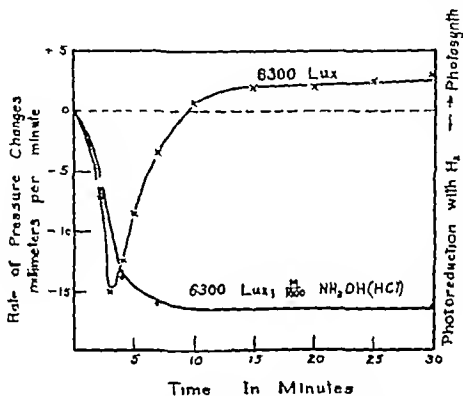


FIG 5 Protection of photoreduction by hydroxylamine against the return to photosynthesis under the influence of excessive light intensity

readings. Thus the rate of photoreduction ( $-H_2$ ,  $-CO_2$ ) is apparently so much larger than that of photosynthesis ( $+O_2$ ,  $-CO_2$ ). The actual rate of carbon dioxide reduction expressed in cubic millimeters of gas absorbed per unit of time is the same in both reactions (see reference 1 and Rieke, unpublished data).

The "protection" given by hydroxylamine, however, is not absolute but only relative to the intensity applied. The higher the intensity at which one intends to maintain photoreduction, the more hydroxylamine one must add. This can be clearly seen in the experiment of Table IX.  $\rightarrow$  signifies the turnback reaction. It is important that even if a turnback is avoided the rate of photoreduction does not rise to the values of the light saturation in aerobic photosynthesis.

Whatever intensities or hydroxylamine concentrations are used, the rate of photoreduction at saturation intensities never reaches much more than 200 per cent of the rate found at the "threshold" intensities without poison. As a complication now enters the sensitivity of photoreduction against higher concentrations of hydroxylamine. The rate of the "protected" reaction at the "threshold" intensity is often lower than that of the unprotected one. Yet the early saturation is not likely to be due to this complicating factor. Such a result supports rather the view that the absorption of hydrogen is the reaction limiting the rate of photoreduction.

TABLE IX

*Stabilizing Effect of Hydroxylamine on Photoreduction*

0.023 cc of cells of *Scenedesmus D<sub>3</sub>* in 3 cc of  $M/30$   $KH_2PO_4$ . Gas phase 8 vol per cent  $CO_2$  in  $H_2$ . 200 or 1000 watt incandescent lamp. Temperature  $25^\circ$ . Poison added in the dark anaerobically 12 hours before exposure. Rate of manometric pressure changes in  $\frac{mm}{min}$  after 5 to 10 minutes illumination at different intensities.

Molar concentration of $NH_2OH \times (10^{-4})$	0	4	10	20
Light intensity	Rates in $\frac{mm}{min}$			
<i>lux</i>				
1500	-4.5	-4.0	-3.7	-3.0
2900	-3.6 $\rightarrow$ +0.7	-6.3	-6.3	-5.1
4500	+1.1	-7.0 $\rightarrow$ 0.0	-8.0	-7.1
6600	+1.25	0.0	-9.0	-8.0
22000	+2.6	0.0	-7.4 $\rightarrow$ 0.0	-7.5

Some emphasis has to be put on the fact that the turn is not prevented completely by concentrations of hydroxylamine which suffice for a total inhibition of photosynthesis. In Table IX, it can be seen that a further increase in intensity may eventually overcome the "protecting" influence of hydroxylamine. Photoreduction comes to an end, but so does photosynthesis. The eventual turnback in presence of hydroxylamine ends in a complete inhibition of any measurable carbon dioxide reduction.

(d) *Turnback in Presence of Dinitrophenol*—There is some similarity in the action of dinitrophenol with that of hydroxylamine described in the preceding paragraph in that it delays the turnback in excessive light. This effect, however, is obtained only at the price of a very strong inhibition of the rate of photoreduction. Even then, the "protection" hardly stands up against any prolonged irradiation at moderate intensities (see Fig. 6). The coordinates of Fig. 6 are similar to those of Fig. 5. Positive pressure changes above the

zero line represent photosynthesis, negative pressure changes below, photo-reduction. Once the turn has taken place also in the poisoned algae the evolution of oxygen commences at the reduced rate still allowed by the particular concentration of dinitrophenol.

(e) *Carbon Monoxide Acceleration of the Turnback Reaction*—Since CO is a very effective inhibitor for all reactions concerning the hydrogen transfer by the hydrogenase system, it is not surprising that the algae return quite readily to photosynthesis when this poison is present (Table VI). Carbon monoxide has apparently no influence at all upon the rate of oxygen evolution or any

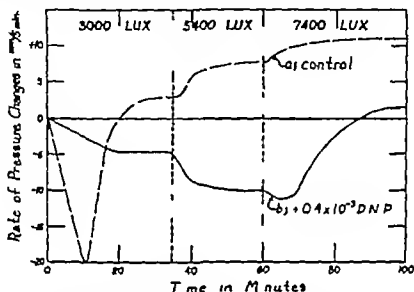


FIG 6 Inhibition of photoreduction and of the return reaction by dinitrophenol in *Scenedesmus*. 0.027 cc. of cells in 4 cc. of  $\frac{1}{30}$   $\text{KH}_2\text{PO}_4$ . Concentration of D.N.P.  $0.4 \times 10^{-3} \text{M}$  added 12 hours before experiment.

other phase of normal photosynthesis. This distinguishes it from all the other poisons discussed above.

#### CONCLUSIONS AND THEORY

It is rather fortunate that the four different substances (cyanide, hydroxylamine, dinitrophenol, carbon monoxide) manifest their specific inhibiting power upon enzymatic reactions in so obviously different ways when applied to the study of photoreduction in algae. The amount of information thus gained is considerable as compared with the existent knowledge about the effect of poisons on normal photosynthesis. The essential conclusions which have to be drawn on account of the evidence now available are as follows.

*Experiments with Cyanide*—Cyanide has an affinity for at least two different catalysts connected with photosynthesis in algae like *Scenedesmus*, *Rhodospirillum rubrum*, *Ankistrodesmus* and probably more species. 1) Cyanide inhibits the utilization of carbon dioxide under aerobic as well as anaerobic

conditions Weller and Franck (6) and Rieke (7) have shown that the truly photochemical process, which can be studied in flashing light, is not sensitive to cyanide. A dark reaction involving carbon dioxide, perhaps the initial reversible fixation (Ruben (4)) is at present the most probable partial reaction which we may consider as affected by cyanide. 2) Cyanide inhibits the adaptation to photoreduction and in strong light or in presence of traces of oxygen hastens the turning back of photoreduction to normal photosynthesis. To avoid contradictions one has to assume that cyanide reacts with a substance which is essential for the function of the hydrogenase system. As long as this substance or catalyst stays oxidized, photoreduction cannot proceed, and cyanide tends to keep this substance in the oxidized state (perhaps reacts with it only in the oxidized form). Compare the literature on the Pasteur effect (23) and the Pasteur enzyme (24).

*Experiments with Hydroxylamine*—Hydroxylamine also produces multiple effects. First of all, it inhibits the release of molecular oxygen in photosynthesis. Then it prevents adaptation to photoreduction. Further it protects photoreduction against the internal re-oxidation under the influence of excess light. Finally, in larger concentrations, it limits the rate of photoreduction, without, however, inhibiting it completely.

It is necessary and at the same time sufficient to assume two reactions in the overall mechanism which are inhibited by hydroxylamine in order to give a satisfying picture of these four observations. Since small amounts of hydroxylamine can prevent any and all formation of free oxygen without inhibiting the reduction of carbon dioxide, we conclude that an enzyme involved in the liberation of oxygen has a particularly high affinity for hydroxylamine. From the fact that inactivation by light is still possible while the evolution of free oxygen is prevented in poisoned cells it follows that the inactivation of the hydrogenase system is due not to oxygen but to a precursor, let us say a "peroxide". On the other hand, hydroxylamine in higher concentrations "protects" against the turnback while the reduction of carbon dioxide still continues. This shows that not only the splitting of the "peroxide" but also its formation can be inhibited by this poison, and further that the reduction of the oxidized photoproducts by hydrogen does not necessarily include the intermediary formation of this "peroxide". As compared with the "peroxide" the first oxidized photoproducts must be relatively unstable and harmless. Otherwise the photochemical mechanism would show some change or inactivation upon prolonged irradiation at saturation intensities in presence of hydroxylamine.

*The Combined Effect of Hydroxylamine and Cyanide*—In Table II of Part I an instance has been presented where the combined influences of hydroxylamine plus cyanide on the rate of photoreduction at low light intensities are smaller than that of cyanide alone. This curious phenomenon can now be understood

more clearly. In photosynthesis the effect of cyanide is generally attributed to an inhibition of the carboxylation reaction which changes carbon dioxide into the substrate of the photochemical process. At present we have good reasons to believe that this fixation reaction is not altered when the transition to photoreduction occurs. Hence the sensitivity toward cyanide during the stationary phase of photoreduction should be the same as in photosynthesis. The experiments show clearly that this is not the case. The inhibition by cyanide is always greater than expected. On the other hand, the transition phenomena to and from the hydrogen metabolism are much more sensitive to cyanide than any other observable reaction in the cell. We explain therefore the effect of cyanide on photoreduction by assuming that the reactions essential to the transition processes continue to some extent during the stationary phase. This conclusion is supported not only by the irreversible return to photosynthesis, which nearly always occurs after the addition of cyanide, but also by the experiment shown in Fig. 7. We see that not only the normal return to photosynthesis under the influence of a higher light intensity is inhibited by hydroxylamine, but also the accelerated turnback in cyanide poisoned algae. In spite of the presence of cyanide the rate of photoreduction increases with the light intensity. Yet, while it seems to offer complete protection against the turnback caused by excessive light, hydroxylamine cannot prevent the eventual, though slow, inactivation due to cyanide. These observations allow us to extend the theoretical picture. We assume that in spite of the presence of hydroxylamine a few "peroxide" molecules are formed continually. The "peroxide" molecules oxidize and inactivate some part of the hydrogen transfer- or hydrogenase system. Since the concentration of the "peroxide" is so small, the reverse reaction, probably identical with the 'adaptation,' proceeds fast enough to repair the damage. There is no loss of activity. In presence of cyanide the reduction of the oxidized hydrogenase system is, as we know, inhibited. Consequently, the eventual inactivation of the whole process is inevitable.

*The Effect of Dinitrophenol*—Though similar to the action of hydroxylamine there is little doubt that we have to interpret the effect of dinitrophenol differently. In Part I we have shown that dinitrophenol inhibits the rate of carbon dioxide reduction regardless of whether oxygen is liberated or hydrogen absorbed in the course of the reaction. The slight 'protecting' effect against the turn caused by an accumulation of 'peroxides' in excessive light goes parallel with an inhibition of the rate of photoreduction and is very likely due to the lower rate at which the "peroxides" are now formed. They simply do not accumulate. The effect of dinitrophenol should be compared rather with the 'protection' against the turnback found in absence of carbon dioxide than with the inhibition by hydroxylamine.

*The effect of carbon monoxide* appears to be solely due to an interference with



a constituent in the hydrogenase system and thus has nothing to do with the very reduction of carbon dioxide. The algae possessing a hydrogenase behave in this respect very similar to other microorganisms capable of utilizing molecular hydrogen. The hydrogen fermentation in many of these organisms is sensitive to carbon monoxide (25, 26). The only photochemical metabolism which proved to be influenced considerably by this poison is that of some *Thiorhodaceae* (21).

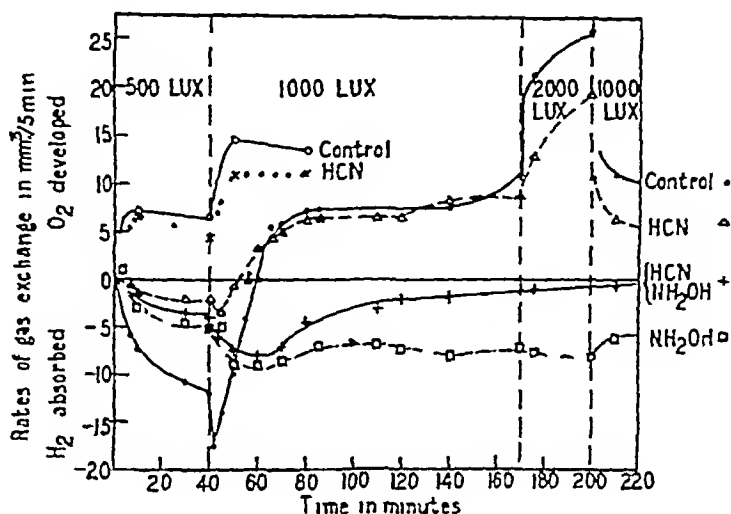


FIG 7 Antagonistic effects of cyanide and hydroxylamine upon photoreduction and the turnback to photosynthesis (Compare Table II)  $\circ-\circ$ , photosynthesis in air  $\times$ , same with  $\mu/5000$  cyanide  $\bullet-\bullet$ , photoreduction (control)  $---\times$ , same with cyanide  $---\square$ , same with  $\mu/1000$  hydroxylamine  $---+$ , same with hydroxylamine and cyanide

#### SUMMARY

1 The effect of poisons upon the photoreduction with hydrogen in *Scenedesmus* and similar algae has been studied. The poisons used were cyanide, hydroxylamine, dinitrophenol, and carbon monoxide, substances known to inhibit more or less specifically certain enzymatic reactions.

2 It was found that quite generally one has to distinguish between the action of poisons upon the photoreduction in the stationary state, once this type of metabolism has been well established in the cells, and their effects on transition phenomena, on the "adaptation" and its reversal, the "turnback" from photoreduction to photosynthesis.

3 Cyanide inhibits photoreduction more strongly than it inhibits photosynthesis in the same algae. It is concluded that the mechanism of oxygen liberation, which is idle in photoreduction, is not very sensitive to cyanide.

4 Hydroxylamine in low concentrations is a powerful inhibitor of photo-

synthesis but has practically no influence on the rate of photoreduction. Consequently, it is assumed that it acts in photosynthesis mainly by inhibiting the evolution of oxygen. Greater concentrations of hydroxylamine clearly inhibit photoreduction, but diminish the rate to about one-half only. A greater degree of inhibition is obtained only by prolonged incubation.

5 Dinitrophenol was found to inhibit strongly the reduction of carbon dioxide, under aerobic as well as under anaerobic conditions. A stimulating effect of dinitrophenol can be demonstrated only with respiration or fermentation, not with photosynthesis.

6 Carbon monoxide interferes with all phases of the hydrogen metabolism in algae. It is supposed therefore to be a specific inhibitor for the hydrogenase system.

7 The "adaptation" to the hydrogen metabolism, which takes place if the algae are incubated anaerobically in hydrogen for several hours, is inhibited completely by very small amounts of cyanide. The adaptation reaction is more sensitive to cyanide than most of the other metabolic processes in the same cell. Correspondingly cyanide enhances the return to aerobic conditions, the "turnback," which occurs under the influence of light of high intensities.

8 Hydroxylamine, applied aerobically, inhibits the adaptation reaction to about the same degree as it inhibits photosynthesis. Photoreduction proceeds after the adaptation in presence of hydroxylamine only at a fraction of the rate that it would have if the poison were added later.

9 Hydroxylamine in concentrations of  $10^{-3}$  M protects the anaerobic metabolism against the return to aerobic photosynthesis which normally occurs under the influence of light of too high intensity. The protection is only relative and the higher the light intensity the more hydroxylamine is needed to keep photoreduction going. Once a "turnback" occurs in presence of much hydroxylamine all photochemical gas exchange comes to an end.



# FERMENTATIVE AND PHOTOCHEMICAL PRODUCTION OF HYDROGEN IN ALGAE

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The investigation reported in this paper rendered mainly two results which are of importance for the analysis of photosynthesis in chlorophyllous plants. Firstly Unicellular algae (*Scenedesmus* and similar species) which under anaerobic conditions are capable of reducing carbon dioxide with molecular hydrogen in the light, will liberate hydrogen slowly in the dark if air is replaced by nitrogen in the surrounding gas phase. This faculty for a hydrogen fermentation in *Scenedesmus* agrees with our knowledge of the dark metabolism of bacteria which utilize hydrogen. These reactions with hydrogen have often been found to be reversible. Secondly Illumination of the fermenting algae enhances the liberation of hydrogen particularly if the substrates of the photochemical reduction process, carbon dioxide and hydrogen, are both absent. The release of hydrogen by the algae under the influence of light occurs at a rate about ten times that of the hydrogen formation in the dark. The rate, however is already limited at low intensities by factors other than the light intensity. Experiments with specific inhibitors, like dinitrophenol, allow us to differentiate between the dark and the photochemical liberation of hydrogen.

Upon return of the photosynthesizing cell to aerobic conditions both phenomena disappear as in the case of photoreduction.

Besides hydrogen and carbon dioxide the anaerobic metabolism of *Scenedesmus* and of similar green algae includes many other substances, notably organic acids formed by fermentation of internal or artificially provided carbohydrates. As far as the nature of these fermentation products is concerned, the present investigation was extended only to the analysis of added glucose and to the identification and determination of lactic acid.

## Methods

A description of the methods used in the present study has been reported elsewhere (Jack Rubin (27)) so that a few summarizing remarks will suffice.

Pure cultures of *Scenedesmus* species *D*<sub>1</sub>, *S* species *D*<sub>2</sub>, and *S. obliquus* were grown at 20°C. in inorganic saline through which a slow stream of 4 per cent CO<sub>2</sub> in air was passed. The culture flasks each containing 200 cc. of medium were inoculated from an agar slant and illuminated for 3 to 4 days with incandescent lamps yielding about 4,000 lux at the bottom of the flasks.

The gas exchange of the algae was determined by Warburg's manometric method

(vessels with two side arms, inner well and vent stopper or with a side arm which can be closed and opened by turning the vessel)

In experiments requiring the addition of glucose bacterial contamination could be prevented for many hours if the manometer vessels were dried immediately before the experiment at 150°C and if the algae were suspended in sterile sugar and buffer solutions. In a series of fifty experiments only two were found contaminated with bacteria. The pure nitrogen, and nitrogen mixtures, used to replace air were passed over finely divided copper at 500°. Hydrogen from the Ohio Chemical Company was used without further purification.

We used as light sources 1) a sodium lamp, its radiation as measured with a photonic cell calibrated against a thermopile is expressed in ergs/cm<sup>2</sup>/sec, 2) several incandescent lamps. The intensity of illumination with these lamps is expressed in lux.

The identity and quantity of the gases involved in the metabolism of the algae have been determined with the familiar reagents introduced directly into the manometric vessels: carbon dioxide with potassium hydroxide solution, oxygen with alkaline pyrogallol, hydrogen by absorption with palladium black and decoloration of methylene blue in presence of platinum.

The total amount of organic acids formed in fermentation was measured by titration of the remaining bicarbonate in the medium. Glucose was determined by a modification of the Shaffer-Hartmann method (28), lactic acid was determined colorimetrically with *p*-hydroxydiphenyl, the intensity of absorption measured with a photocell (29).

## PART I

### *The Formation of Hydrogen in the Dark*

It is well known that green plants begin to ferment as soon as the oxygen partial pressure in the surrounding medium drops below the value necessary to maintain the Pasteur effect (inhibition of fermentation by oxygen). In the dark a suspension of algae in water or in an acid buffer will produce carbon dioxide when placed in an atmosphere of nitrogen. If we place the algal suspension in a Warburg vessel, the evolution of carbon dioxide can be followed manometrically. With a 5 per cent solution of potassium hydroxide in a side arm of the vessel, no pressure changes are visible. This indicates that no other gas is liberated in fermentation but carbon dioxide. All this is common knowledge. With several strains of the algae *Scenedesmus* we observed, however, that under the conditions described a Warburg manometer containing a potassium hydroxide solution in the side arm showed the development of a gas different from carbon dioxide. A comparison between identical samples of a *Scenedesmus* suspension fermenting in absence and in presence of an absorbent for carbon dioxide revealed that the new gas begins to appear after a prolonged anaerobic incubation of about 2 hours at 25°. This gas is formed in addition to, and independent of, the evolution of carbon dioxide which is liberated from the very beginning.

Fig 8<sup>1</sup> demonstrates the course of the anaerobic gas production by *Scenedesmus* in absence (curve *a*) and presence (curve *b*) of potassium hydroxide. The vessels used were of equal volume (15.4 and 15.0 cc.) The broken line is obtained as the difference between curves *a* and *b*. It indicates that curve *a* would continue as a straight line except for the additional production of gas beginning after 2 hours of fermentation. Experimentally the rate of fermentation is found to continue unchanged when the vessel showing normal fermentation contains a sufficient amount of palladium black.

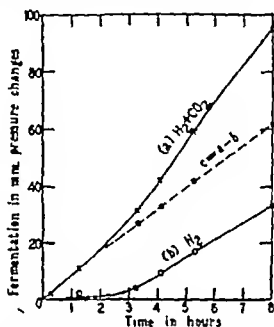


FIG 8 Liberation of molecular hydrogen during fermentation in the alga *Scenedesmus*. 0.027 cc. of cells in culture medium with 0.01 M phosphate buffer pH 6 containing 0.2 per cent glucose. Temperature 25°. Curve (b) KOH solution in side arm of vessel, absorbing  $CO_2$ .

The appearance in *Scenedesmus* of a new metabolic reaction after a fermentation period of 1 or 2 hours at 25° agrees with the observations on the photo-reduction with hydrogen. The latter becomes possible in *Scenedesmus* not simply by substituting hydrogen for air but only after a suitable anaerobic incubation period.

This similarity suggested that the same system, which in its reduced state enables the cells of *Scenedesmus* to utilize hydrogen, was also responsible for the production of the gas not absorbed by potassium hydroxide. The data contained in Table V are, in our opinion, evidence that this gas is hydrogen. If equal amounts of *Scenedesmus* cells fermenting in the same medium are compared the difference in the amount of gas produced in absence and in presence of palladium black is equal to the gas production found in presence of potassium hydroxide.

<sup>1</sup> Figs. 1-7 and Tables I-IX belong to the preceding paper (I)

Qualitatively it can be shown that the gas decolorizes a methylene blue solution in contact with platinum

The gas is not nitrogen, methane or ethylene because none of these three substances will react anaerobically with palladium in the way observed. It is not oxygen because it is not absorbed by alkaline pyrogallol. Nor is it carbon monoxide because we have found carbon monoxide to be a specific inhibitor for this reaction and all metabolic reactions in *Scenedesmus* involving molecular hydrogen<sup>2</sup>

TABLE X

*Production of Hydrogen during the Fermentation of Scenedesmus (Species D<sub>1</sub>)*

0.095 cc of cells in 3 cc of 0.025 M phosphate buffer pH 6.2 Suspension contains 0.07 per cent glucose Temperature 36° Gas phase N<sub>2</sub>

	Time	1	2	3
		CO <sub>2</sub> measured in presence of Pd	H <sub>2</sub> measured over KOH	H <sub>2</sub> measured as difference between pressure changes in vessels with and without Pd
		Rates of gas exchange in $\frac{\text{c.mm}}{30 \text{ min}}$		
Dark (after 40 min)	min			
	30	+28	+10.4	+8.1
	60	+20	+7.5	+10.5
	90	+24	+10.4	+7.2
	170	+21	+7.9	+7.9
	200	+18	+4.5	+5.9
Light, $\lambda 5890\text{\AA}$				
I = $3 \times 10^3$ ergs/cm <sup>2</sup> /sec	30	+20	+9	+5.5
I = $12 \times 10^3$ ergs/cm <sup>2</sup> /sec	120	+13 → +27	+22 → +25	+0.3 → -7.5

\* In the light the method used for the data in column 3 ceases to be useful

**Autofermentation** —In order to observe a measurable fermentation it is not necessary to add a substrate to the cell suspension. The source of hydrogen is one of several unknown reserve substances stored in the cell. The rate of the fermentative evolution of hydrogen in *Scenedesmus* declines with time. Starved cells, which have been allowed to respire for a long time in the dark, show a low rate of fermentation and of hydrogen formation with a tendency toward a further decline with time. Cells which have assimilated carbon dioxide in the light for several hours give the best yield of autofermentation including the liberation of hydrogen.

<sup>2</sup> Since the manometric hydrogen determination is accurate only to about 5 per cent, there is ample space for the discovery that still another gas is formed in traces during fermentation.

*Dependence of the Evolution of Hydrogen on the Hydrogen Partial Pressure —*

In the experiments on photoreduction or on the oxyhydrogen reaction the algae were incubated not in nitrogen but in hydrogen for a period of several hours previous to the experiments. During this time they invariably absorbed hydrogen sometimes in considerable amounts. Not only in presence of an absorbent for carbon dioxide, but also if the carbon dioxide of fermentation was allowed to accumulate, the balance of the overall gas exchange was negative. The algae fermenting in hydrogen absorb more gas than they produce.

TABLE XIa

*Fermentation of Scenedesmus obliquus in Hydrogen and in Nitrogen*

0.105 cc. of cells in 3 cc. of 0.05 M phosphate buffer pH 6.2. 0.4 cc. of 2 per cent KOH solution on a piece of paper in the side arm of the vessels. Turning the vessels around their vertical axis closes or opens the side arms. (See Fig. 14 in Paper III.) Temperature 25°C. 2 hours of fermentation in  $N_2$  in both vessels before start of experiment. Vessel 1 filled with  $H_2$ .

		1		2	
		Side arm closed	Side arm open	Side arm closed	Side arm open
Vessel constant for CO <sub>2</sub>		1 49	1 74	1 56	1 83
Vessel constant for H <sub>2</sub>		1 25		1 32	
		H <sub>2</sub>		N <sub>2</sub>	
	Time	Pressure changes in mm.			
Dark	12 hrs. 30 min.	-81	-73	+112	-71

*Computation of Gas Exchanges*

$$\begin{array}{l|l}
 \text{In } H_2 & \text{In } N_2 \\
 73 \times 1.74 = +127 \text{ c.mm. } CO_2 & 71 \times 1.83 = +130 \text{ c.mm. } CO_2 \\
 \left(-81 - \frac{127}{1.49}\right) \times 1.25 = -207 \text{ c.mm. } H_2 & \left(112 - \frac{130}{1.56}\right) \times 1.32 = +37 \text{ c.mm. } H_2
 \end{array}$$

If we compare this with the opposite results on the fermentation in nitrogen, it is obvious that the presence or absence of hydrogen in the gas phase must influence the activity of the hydrogenase system.

Table XIa and b presents the results of an experiment in which algae of the same culture were brought into two vessels, and one part was left to ferment in pure nitrogen, the other part in pure hydrogen. To make sure that conditions were comparable in both vessels the hydrogen was introduced into one vessel after a preliminary fermentation period of 2 hours in nitrogen during which the gas exchange in both vessels was equal. The design of the vessels used (described in Paper III) is such that after a certain time a side arm containing potassium hydroxide is opened. In this manner the carbon dioxide released from the slightly acid algal suspension during the interval is measured and compared with the total gas exchange.



In this particular experiment the amount of carbon dioxide formed under nitrogen was equal to that liberated under hydrogen. Simultaneously the algae in nitrogen had produced hydrogen in a quantity equal to about one-third the amount of carbon dioxide formed while the algae in hydrogen had absorbed hydrogen in an amount about twice that of the carbon dioxide. There are as yet no experiments available which allow one to decide whether a high partial pressure of hydrogen reverses the process of hydrogen production or whether both liberation and absorption proceed simultaneously and independently. A parallel experiment with algae suspended in bicarbonate showed that the fermentative formation of acids was the same in hydrogen as in nitrogen (Table XIb). The first possibility, the existence of a true equilibrium between bound and free hydrogen, would offer an explanation for the

TABLE XIb

*Acid Formation during Fermentation in Hydrogen and in Nitrogen*

0.114 cc of cells of *Scenedesmus D<sub>1</sub>* in 3 cc of  $3.3 \times 10^{-2}M$   $KHCO_3$  Temperature 25°  
40 minutes fermentation in  $N_2$  before experiment.

No. and volume of vessels	(1) 18.6 cc. (2) 18.0 cc.	(3) 18.4 cc (4) 18.2 cc.
Initial bicarbonate content in each vessel 212 c mm $CO_2$		
Gas phase	$H_2$ , 4 per cent $CO_2$	$N_2$ , 4 per cent $CO_2$
Total pressure change in 15 hrs, mm	-94      -94	+167      +176
Final bicarbonate content in c mm $CO_2$	114      117	116      116
Acid formed in $H_2$ and $N_2$	96 c mm	96 c mm

fact that sometimes the determination of hydrogen formation by difference in presence and absence of palladium renders results not checking with the direct determination of hydrogen formed in presence of alkali. The accumulation of hydrogen in the vessels without palladium might gradually inhibit the evolution of hydrogen—which proceeds uninhibited where the hydrogen is continuously removed.

*Influence of Glucose and of Other Organic Substances upon the Hydrogen Formation*—Of many organic substances added to the cell suspension only glucose was found to increase immediately all phases of the anaerobic metabolism (Table XII). Other monosaccharides were effective after a definite time lag. The phosphates of glucose (mono- and di-), of glycerol, of glyceric acid, etc., were all without effect. They probably did not penetrate to the proper places. Pyruvic and lactic acids gave positive results, but the difficulties, with respect to permeability and to the inhibiting properties of these acids, leave it an open question whether their effect is in any way to be looked upon as a direct one.

The experiments shown in Fig 9 and in Tables XII-XIV demonstrate the accelerating effect of added glucose. As yet we have found no evidence that formate could be an intermediate in the course of the hydrogen production (Compare reference 25, page 96)

*Influence of the Hydrogen Ion Concentration*—Contrary to the behavior of respiration and photosynthesis, the rate of fermentation in the algae is clearly dependent on the hydrogen ion concentration in the suspension. The optimal hydrogen ion concentration varies with other conditions influencing the fermentation, *e.g.* the absence or presence of added glucose and the

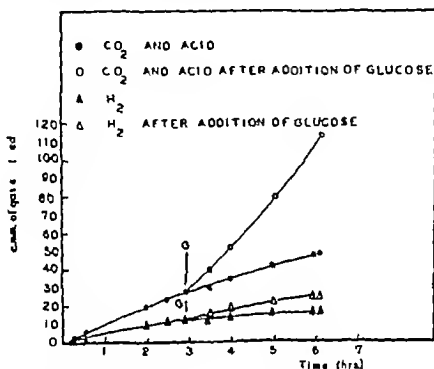


FIG. 9 Increase of fermentation and of hydrogen production in *Scenedesmus* upon addition of glucose. Final sugar concentration 0.06 per cent.

time elapsed since the beginning of the anaerobic period (See Fig 10) Such variations of pH optima are already known in bacterial metabolism (25) For our purposes a pH between 6 and 7 appeared best

*The Relative Amounts of Carbonic and Organic Acids Produced in Fermentation*—Whereas it could be established beyond doubt that added glucose enhances the production of hydrogen, numerous experiments failed to reveal a clear and uniform relationship between the amounts of the different fermentation products and of the glucose added. The hydrogen production remains rather small compared with the quantities of potential hydrogen donors available (See Table XIV)

There is also hardly a constant relation between the volume of hydrogen evolved and that of carbon dioxide gas or of fixed acids produced during a given fermentation period (Tables XI, XII, XIV and XV) This is not too

TABLE XII

*Influence of Glucose on the Hydrogen Fermentation*

0.048 cc of cells of *Scenedesmus D<sub>1</sub>* in 3 cc of 0.01 M KHCO<sub>3</sub>. Gas phase N<sub>2</sub>, 4 per cent CO<sub>2</sub>. Temperature 36°. Gas exchange computed from experiments in presence and absence of palladium black, which absorbs all hydrogen. The data represent the average of three (six) parallel experiments.

	Time elapsed since start of experiment	Rate of gas produced in $\frac{\text{c mm}}{30 \text{ min}}$		Ratio $\frac{\text{CO}_2}{\text{H}_2}$
		H <sub>2</sub>	CO <sub>2</sub>	
	<i>min</i>			
(a) Autofermentation	70	3.5	7.8	2.2
	100	3.0	5.5	1.8
	130	2.0	6.6	3.0
(b) Glucose added (0.1 per cent)	160	4.5	11.3	2.5
	220	10.7	18.2	1.7
	280	6.3	18.6	2.9
	400	4.6	14.4	3.1

TABLE XIII

*Influence of Carbohydrates on the Rate of Hydrogen Production*

0.05 cc of cells of *Scenedesmus obliquus* in 4 cc of 0.05 M phosphate buffer pH 6.5. Different sugars added aerobically. Final concentration in medium 0.12 per cent. Side arms of all vessels contain 0.2 cc of potassium hydroxide-pyrogallol solution. Temperature 25°. Gas phase N<sub>2</sub>.

	Time	Control	Glucose	Galactose	Mannose	Hexosed- and hexose mono-phosphate
		Rate of hydrogen production in $\frac{\text{c mm}}{10 \text{ min}}$				
Dark	140 min	0.7	1.2	0.7	1.0	0.6
Light 3700 lux	10 min	6	10	7	7	8
Dark	11 hrs	0.3	0.7	0.7	0.7	0.3
Dark	20 min	All vessels flushed with N <sub>2</sub> to remove H <sub>2</sub> of fermentation				
Light 1700 lux	5 min	6	18	10	12	8
Light 1700 lux	15 min	4	11	5	5	4

surprising if we remember that the hydrogen fermentation needs some additional adaptation before it can start, as compared with the ordinary fermentation which is regulated simply by the oxygen partial pressure (Pasteur effect). It is very probable that two or more independent fermentation

TABLE XIV

*Acid Formation during Fermentation of Scedermus (Strain D<sub>1</sub>)*

0.06 cc. of cells in 3 cc. of  $0.5 \times 10^{-3}M$   $KHCO_3$ . Temperature 36 Gas phase  $N_2$ , 4 per cent  $CO_2$ . Duration of experiment 5 hours, after 1 hour of preliminary anaerobic incubation. All data expressed in cubic millimeters normal gas.

	Initial	Final	Difference	Initial	Final	Difference
(a) Glucose expressed in c. mm. normal gas (180 mg. equivalent 22400 c.mm.)	—	—	—	Filled in 620 found 581	361 344	229
(b) Acid formed = change in bicarbonate	201 205	110 114	91	203	59 59	144
(c) Lactic acid formed	3 6	5 3 7 2	2 6	3 6	31 33	28 4
(d) Total gas formed ( $H_2 + CO_2 +$ decomposed bicarbonate)	0 0	175 175	175	0 0	291 297	294

Adding glucose results in the production of 53 c.mm. more organic acid of which 25 c.mm. = 50 per cent are lactic acid. Only 3 per cent of the 91 c.mm. acid formed in fermentation of intracellular material are lactic acid.

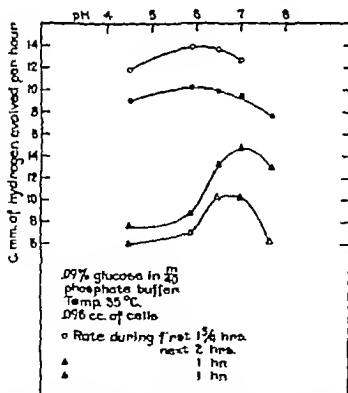


FIG. 10. Influence of the hydrogen ion concentration in the medium upon the rate of hydrogen liberation in the dark

reactions can proceed simultaneously in *Scenedesmus*, perhaps, at quite different locations in the cell. We shall demonstrate in Part II that the hydrogen-liberating system may be linked with the photochemical activity of the plants, and the latter has its seat in the chloroplasts. On the other hand, it is known that the non-chlorophyllous parts of a plant have a definite fermentative capacity of their own. The situation is similar to that found with respiration in *Chlorella*. Autorespiration of photosynthetic products has different properties from that of substrates artificially supplied (Emerson (30)). As it was (13) pointed out before, one should consider in such a case the possibility

TABLE XV

*Effect of Light upon the Liberation of Molecular Hydrogen in the Alga Scenedesmus*

Algae grown with 1 per cent glucose    Suspended in phosphate buffer pH 6.5    Gas phase  
N<sub>2</sub> (free of CO<sub>2</sub>)    Temperature 25°    Preceding dark time 12 hours

No	1	2	3	4
Volume of vessels, cc	18.3	18.0	18.4	18.2
Volume of liquids, cc	3.0	3.2	3.0	3.2
In side arms	—	KOH	Pd	KOH, Pd
	Rate of pressure changes in $\frac{\text{mm}}{\text{hr}}$			
Dark	+16	+2	+13	+0.3
Light. 740 lux	+31	+15	+18	+2
Light minus dark	+15	+15	+2	+1
Dark	+17	-2	+18	+1
Light. 1500 lux	+21	+15	+18	0
Light minus dark	+4	+17	0	-1

not only of different catalytic systems but also of a separate location in the cell of these systems.

In some experiments we tried to shorten the adaptation time and to increase the rates by raising the temperature from 25 to 35°. Although fermentation continues at the higher temperature for many hours the photosynthetic capacities of the cells suffer an irreversible damage. Such cell samples should not be used in combined dark and light experiments. One might use inhibitors to separate and isolate the different fermentation processes from one another. Kempner, for instance, detected that the hydrogen fermentation of *B. butyricum* was stopped by carbon monoxide (26). Later Kubowitz (31) showed that this effect is not a true inhibition of glucose decomposition but a change from hydrogen fermentation to lactic acid formation.

Our experiments with inhibitors reported below gave results resembling those of Kempner and Kubowitz with butyric acid bacteria. Since in *Scene-*

*desmus* at least two types of fermentation reactions seem to occur simultaneously in varying proportions, more work than we have devoted to this problem is needed to disentangle the fermentation reaction and to establish the stoichiometry of the reaction yielding hydrogen

*The Formation of Lactic Acid*—Besides other acid compounds of yet unknown constitution, lactic acid appears only in very small amounts during autofermentation of natural reserve material, as shown in Table XIV. On the other hand, this acid often accounts for 50 per cent of the fermentation acids formed after adding glucose to a cell suspension. Barker found in the colorless heterotrophic alga *Prototheca* that glucose is decomposed up to 95 per cent into lactic acid.

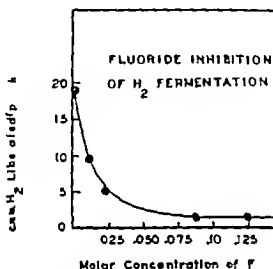


FIG. 11 Inhibition of hydrogen production by fluoride. Abscissa molarity of fluoride ion

Attempts to inhibit only the lactic acid formation with fluoride were not successful. The dark formation of hydrogen is also sensitive to fluoride (Fig. 11).

$Q_{H_2}$  (Dark)—The familiar quotient  $Q_{H_2}$  (dark) =  $\frac{\text{cmm } H_2}{\text{hrs} \times \text{mg dry cells}}$  was found to attain values not larger than 1. The weight of the dry material in milligrams was assumed (according to earlier measurements) to be 1/5 of the number of cubic millimeters of wet cells tightly packed by 15 minutes centrifugation at 3,000 rev./min.

#### CONCLUSIONS

From the evidence presented above we have to conclude that those algae which have the capacity for photoreduction with hydrogen contain a hydrogenase capable of functioning independently of any photochemical process. Hydrogenase functions here as a catalyst establishing an equilibrium between

molecular hydrogen and various intracellular hydrogen-transferring systems. The potential of these hydrogen-transferring systems and the partial pressure of free hydrogen determine whether hydrogen will enter or leave the algal cell (Compare Farkas (33) )

## PART II

### *The Photochemical Production of Hydrogen*

Under the proper anaerobic conditions, *Scenedesmus* and similar algae can utilize hydrogen for the reduction of carbon dioxide. We expected, therefore, that the hydrogen production by fermentation in the dark would disappear completely upon illumination. This is true indeed if carbon dioxide is present in sufficient amounts, either from the beginning of the experiment or if it accumulates during fermentation periods. When the algae are irradiated, hydrogen fermentation gives way to photoreduction and eventually to normal photosynthesis. Shape and duration of these transitions depend on the intensity applied and the conditions of the plants (see Paper I)

In the absence of carbon dioxide (e.g. KOH in side arm of the manometer vessel), the irradiated cells can make use only of that small part of the carbon dioxide formed in fermentation which does not escape into the gas phase. In the light, therefore, there will be neither an appreciable photoreduction nor a return to aerobic conditions (which depends on the presence of sufficient carbon dioxide), nor any photo-oxidation (which depends on available oxygen). Under these conditions light absorbed by the cells might be transformed into heat. With *Chlorella*, for instance, no observations have been made which would contradict such an assumption. The situation is surprisingly different, however, with the algae possessing a hydrogenase system. Irradiating *Scenedesmus* under anaerobic conditions ("reduced state") in absence of carbon dioxide, we observed a measurable photochemical activity. This new reaction is a photochemical production of gas which continues for hours, though at a rapidly declining rate. The gas liberated under the influence of light is hydrogen, and the algae produce it at a rate up to ten times the dark fermentation (Tables XV, X-XIII, XVI)

The evidence for the gas to be hydrogen is the same as already discussed in Part I. The gas is not absorbed by alkali, nor alkaline pyrogallol, which remains colorless (Table XVI). The decisive test that the gas produced under the conditions described is not the oxygen of photosynthesis is furnished by the photochemical reaction following the introduction of a mixture of hydrogen and carbon dioxide into the vessel containing the irradiated algae. If the gas produced photochemically by the algae were the oxygen of normal photosynthesis, they would continue to produce oxygen, probably at a much higher rate. What actually happens is that the algae cease to produce gas and turn to photoreduction with hydrogen without requiring the usual "adaptation

period" (Fig 12) This is proof that the photochemical mechanism in these organisms has remained in the "reduced state" where no liberation of oxygen occurs Furthermore, the gas in question is completely absorbed by palladium black (Table XV) <sup>1</sup>

In an atmosphere of hydrogen, the photochemical evolution of hydrogen (in absence of the reducible substrate  $\text{CO}_2$ ) is inhibited as compared with the reaction in nitrogen Hydrogen is often released from the cells, however, in spite of the presence of a little hydrogen and carbon dioxide Fig 12 shows that whether we observe photoreduction or hydrogen evolution depends on

TABLE XVI

*Photochemical Development of Hydrogen in Scenedesmus*

0.04 cc. of cells in 0.05 M phosphate buffer pH 6.5 Temperature 38° Gas phase  $\text{N}_2$  In side arms of vessels 0.2 cc. of 7 per cent KOH solution.

	1	2	3
Volume of vessels, cc.	16.0	15.0	15.4
	Pressure changes in mm.		
Dark, 240 min.	+27	+41	+30
Light, 240 min. 1000 lux	+74	+66	+66
0.1 cc. of a concentrated pyrogallol solution added to KOH solution in side arm	No color	Trace yellow	No color
	No significant pressure changes		
$\text{O}_2$ introduced into vessels, mm	30	75	50
Color change in side arm 5 min. later	Yellow	Brown	Yellow
Gas absorbed in the dark during first 15 min. after $\text{O}_2$ has been introduced, mm	-9	-26	-17

the light intensity In the experiments, one of which is represented in Fig. 12, the algae had been fermenting for several hours in nitrogen. About 0.2 vol per cent of hydrogen had accumulated on account of this fermentation All carbon dioxide diffusing into the gas phase had continuously been absorbed by potassium hydroxide, while it was allowed to accumulate in a parallel experiment not shown here Only during the last phase, where some hydrogen had been introduced into the vessels, a difference between the reaction of both samples of cells was found This consisted in a less rapid decline of the rate of photoreduction in the vessel which had no potassium hydroxide solution in its side arm We learn from the time course of the gas exchange in Fig 12 that an absorption of hydrogen at very low light intensities can change instantaneously into a liberation of hydrogen at higher light intensities For 20 minutes a  $Q_{\text{H}_2}$  of +4 is maintained That this evolution of gas is not a



turnback to photosynthesis (which is not likely to occur in absence of carbon dioxide) is demonstrated by the photochemical absorption of added hydrogen immediately afterwards

*The Influence of Hydrogen Donors*—The photochemical evolution of hydrogen depends clearly upon the presence of suitable hydrogen donors in the cell. The same circumstances which improve the dark hydrogen fermentation, addition of glucose or a previous intense photosynthesis, increase the yield of

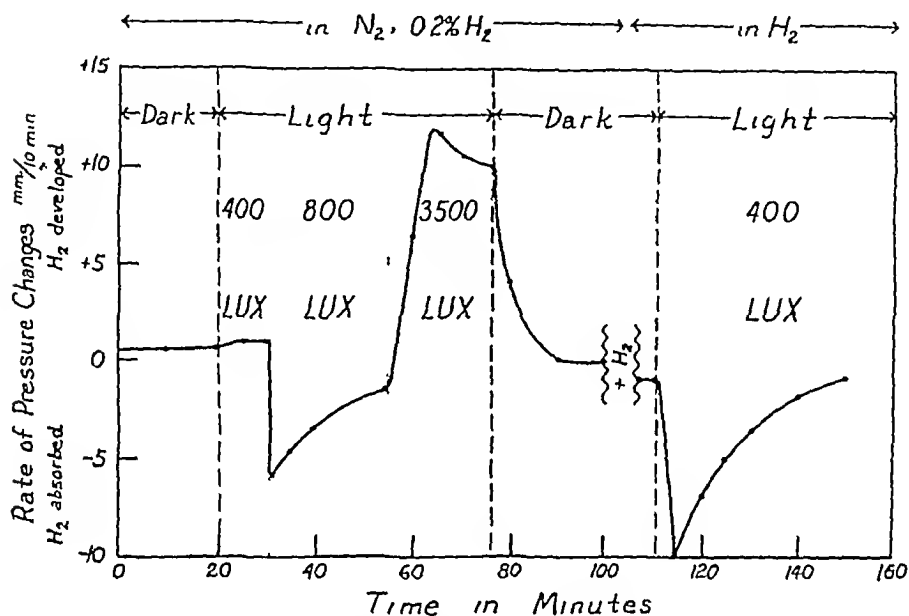


FIG 12 Rates of photochemical absorption and evolution of hydrogen in absence of carbon dioxide at different intensities. 0.074 cc of cells of *Scenedesmus D<sub>3</sub>* in 0.05 M phosphate buffer pH 6.2. Side arm of vessel contains KOH. Gas phase N<sub>2</sub> later H<sub>2</sub>. Preceding dark period 20 hours in N<sub>2</sub>. During this time the algae have formed H<sub>2</sub> amounting to 0.2 per cent of the available gas volume. Temperature 25°

the photochemical hydrogen production. The rate decreases with time, and we have often observed an apparent exhaustion of the supply of hydrogen donors. This indicates that dark reactions constitute factors limiting the overall rate of the process.

In spite of the fact that the same conditions which favor the hydrogen fermentation also support the photochemical evolution of hydrogen we must emphasize that the new light reaction is not simply an acceleration of the dark fermentation which continues unchanged. Table XV contains data which make this fact clear. It is important for the understanding of the photochemical reaction that the effect of light is apparently restricted to an increase of the production of hydrogen alone, and does not increase simultaneously the

output of free carbon dioxide and of organic acids. This will become even more apparent in Part III concerning the effect of poisons on the photochemical hydrogen production

*Anaerobic Induction Periods*—The tendency of the algae to liberate hydrogen upon illumination after an anaerobic fermentation period prevails even in presence of carbon dioxide, though only for a short time. Gaffron (18, 20) has described irregularities of the gas exchange of illuminated algae preceding normal photosynthesis when the plants had been kept for several hours in nitrogen. With algae of the *Scenedesmus* type, the gas exchange during the anaerobic induction period consists first of a vigorous evolution of gas lasting from 1 to 3 minutes followed by a gas absorption which then turns into normal photosynthesis. (Compare Figs 1 and 2 in reference 20.) Since at that time it was found that no oxygen is formed during the first two phases of the anaerobic induction period, and since Blinks and Skow, working with other species of algae, reported an initial carbon dioxide gush during the first seconds of illumination, Gaffron interpreted the first momentary gas production as a liberation of carbon dioxide. We have repeated these experiments in the presence of palladium black. In all cases the greater part, and in some cases all of the initial gas evolution, disappeared in presence of this absorbent for hydrogen, while the second and third phase of the anaerobic induction period remained more or less as described. In our opinion the correct interpretation of the anaerobic induction period with algae containing a hydrogenase system is as follows. After a fermentation period of many hours the reduction of carbon dioxide is initially inhibited, possibly on account of organic acids, and the plants react as if carbonic acid were absent. That is, they start to produce hydrogen. With the beginning of carbon dioxide reduction, the evolution of hydrogen ceases and a period of photoreduction with internal hydrogen donors follows. Then, depending on the light intensity used, this is succeeded more or less quickly by normal photosynthesis with the evolution of oxygen. (Compare Fig 13a with Fig 13b and with Fig 1 in reference 20.) According to the recent results of Emerson and Lewis with *Chlorella* (32) some carbon dioxide is released during the aerobic induction period upon illumination of the algae after a longer dark pause. How much  $\text{CO}_2$  is liberated before normal photosynthesis starts depends on the condition of the algae and the concentration of carbon dioxide in the surrounding medium. It is possible, therefore, that the initial evolution of hydrogen during the anaerobic induction period in *Scenedesmus* is accompanied in the same way by varying amounts of carbon dioxide.

$Q_{\text{H}_2}$  (Light)—If we express the rate in  $\frac{\text{cmm H}_2}{\text{hrs.} \times \text{mg dry weight}}$  generally a value of 3 and a maximal value of 7 is obtained. Though the hydrogen liberation in the light is about ten times the rate in the dark (with very large deviations to both sides), it is still very small as compared with the saturation rate

of normal photosynthesis The rate of photoreduction with hydrogen seldom surpasses the order of magnitude of respiration in the same algae, and the

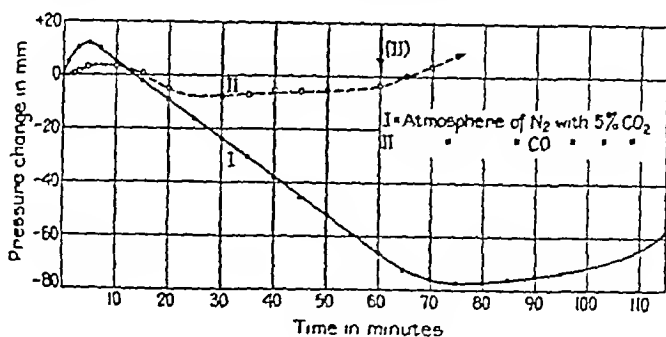


FIG 13a

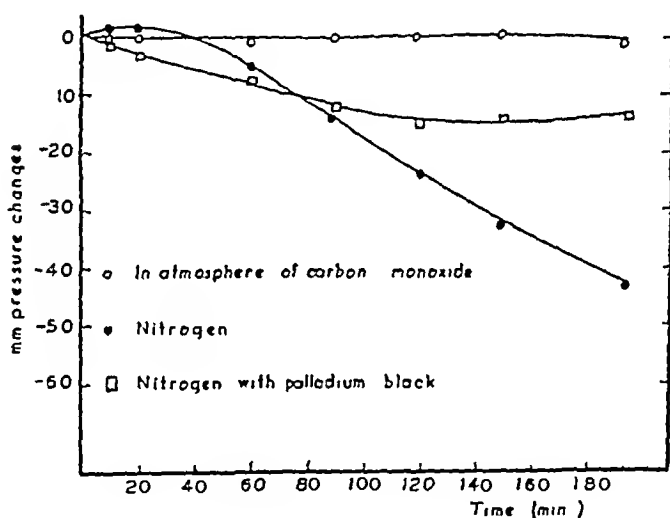


FIG 13b

FIG 13 Gas exchange of *Scenedesmus* during the induction period after several hours of anaerobic incubation in  $N_2$  and its inhibition by carbon monoxide Fig 13a, according to Gaffron in 1935 The initial evolution of gas was interpreted as an "oxygen gush," followed by an internal  $CO_2$  reduction Fig 13b shows a similar experiment In presence of palladium black the initial gas production is not visible The rate of photoreduction appears to be smaller because only intracellular hydrogen donors are available The hydrogen developed during the preceding fermentation period has been absorbed by palladium All anaerobic photochemical gas exchange is inhibited by carbon monoxide

photochemical hydrogen evolution stays mostly below those rates It appears, from studies of the oxyhydrogen reaction in the dark (Paper III), that in all cases the hydrogen transfer systems (including the hydrogenase) are responsible for these slow rates

## PART III

*The Effect of Specific Inhibitors upon the Production of Hydrogen in Algae*

The preceding observations on the hydrogen fermentation and on the photochemical evolution of hydrogen in green algae of the *Scenedesmus* type indicate that both reactions, though enhanced by the same anaerobic conditions, may have only one catalyst in common and are independent of one another in other respects. In this part, we present more evidence supporting the assumption that the hydrogen liberated photochemically may have a different origin than that developed by fermentation. With dinitrophenol as a poison, it is possible to inhibit largely if not completely the evolution of hydrogen in the dark while the photochemical production of this gas continues at the normal or even an increased rate (Tables XVII-XIX)

As described in the preceding paper (Paper I), minute amounts of dinitrophenol influence all phases of the algal metabolism. In agreement with results obtained with other organisms reported in the literature, the inhibiting or stimulating effect is a function not only of the total concentration of dinitrophenol, but also of the hydrogen ion concentration, probably because the free acidic form is the poison proper. Table XVIII shows that  $4 \times 10^{-4}$  M dinitrophenol at pH 6.2 inhibits the dark hydrogen fermentation, while at the same time the lactic acid formation is increased (See also Table XIX.) This appears to be a metabolic change similar to that known to occur in *B. butyricum* (Kempner Kubowitz (26), (31)) where the inhibition of the hydrogen liberation by cyanide or carbon monoxide leads to an increase in lactic acid production. The stoichiometric relations, however, are different inasmuch as the uninhibited hydrogen fermentation in algae is already a smaller fraction of the total anaerobic metabolism than in the bacteria mentioned.

Table XIX shows what happens upon illumination of the poisoned algae in comparison with those kept under normal anaerobic conditions. As in all experiments on photosynthesis etc. we assume the true effect of light to be equal to the difference between the metabolic rates in the light and in the dark. An essential difference of this experiment from that presented in Table XV is that the gas phase contains 4 per cent of carbon dioxide in nitrogen instead of pure nitrogen. The unpoisoned algae will respond to light, therefore, with a photoreduction of carbon dioxide partly at the expense of the hydrogen developed during the previous dark time, partly or mainly at that of the internal hydrogen donors. The pair of manometer vessels containing the poisoned algae present a quite different result. The light effect here is the production of gas which is completely absorbed by palladium, i.e., a production of hydrogen. Hydrogen is liberated photochemically though its fermentative production has been oppressed by the poison. Furthermore, hydrogen appears despite the presence of carbon dioxide. The action of the poison is thus two-

fold Firstly, it differentiates between the mechanisms of hydrogen production in the dark and in the light Secondly, it inhibits the photoreduction

TABLE XVII

*Effect of Dinitrophenol on the Production of Hydrogen in Scenedesmus*

(a) 0.050 cc of cells in 3 cc of 0.05 M phosphate buffer pH 6.2 Temperature 25° Gas phase  $N_2$  In the side arm of the vessels 0.2 cc 7 per cent KOH Light intensity I (sodium line 5890 Å) measured in  $10^3$  ergs/cm<sup>2</sup>/sec

		1	2
Volume of vessels, cc		19.8	21.2
Condition	Observation time	Rate of $H_2$ evolution in $\frac{cc}{10 \text{ min}}$	
	min		
Dark	120	+0.85	+0.70
Poison added	—	—	$2 \times 10^{-4} M$ dinitrophenol
Dark	90	+1.03	$\pm 0$
Light I = 3.0	50	+1.8	+1.9
Dark	80	+0.4	(-0.2)
Light I = 7.5	30	+6.1	+8.1
Light I = 12	15	+9.1	+11.0

(b) 0.04 cc of cells of *Scenedesmus D<sub>3</sub>* in 4 cc of 0.033 M phosphate buffer pH 6.3 In Experiments 2 and 3 the suspension contains 5 mg glucose Temperature 25° Gas phase  $N_2$  (control  $H_2$ , 4 per cent  $CO_2$ ) Preceding incubation time 3 hours

		1	2	3
Volume of vessels, cc		15.0	16.0	15.4
Gas phase Poison added		$H_2/CO_2$ —	$N_2$ —	$N_2$ $0.2 \times 10^{-3} M$ dinitrophenol
	Time	Rate of pressure changes in $\frac{mm}{10 \text{ min}}$		
Dark	40 min	-0.3	+1.5	+0.7
Light 1000 lux	First 10 min	-18	+7	+9
	After 20 min	-27	+3.5	+8
	After 50 min	-26	+4	+7
Dark	10 min	-1	+1	$\pm 0$

going on in the unpoisoned algae In the absence of carbon dioxide only the first effect becomes apparent

It has been shown that photoreduction as well as normal photosynthesis are inhibited by dinitrophenol (Paper I) This substance is probably a specific poison for the transfer of hydrogen to carbon dioxide That gives us an ex-

TABLE XVIII

*Increase of Lactic Acid Formation in Presence of a Concentration of Dinitrophenol Which Inhibits the Dark Hydrogen Fermentation*

0.055 cc. of cells of *Scenedermus D<sub>2</sub>* in 3 cc. of 0.05 M phosphate buffer pH 6.2 Temperature 35 Gas phase N<sub>2</sub> 0.2 cc. of 10 per cent KOH solution in side arms of vessels. Dinitrophenol added anaerobically after 3 hours dark incubation.

Condition	Observation period	Control	$4 \times 10^{-4}$ M dinitrophenol
		Hydrogen liberated in c.mm	
(a) Photochemical (low intensity)	20 min.	9	14
(b) Fermentative (dark)	11 hrs	88	0
		Lactic acid (expressed in c.mm normal gas)	
(c) Lactic acid formation	15 hrs.	5	47

TABLE XIX

*Effect of Dinitrophenol on the Hydrogen Production in Scenedermus D<sub>2</sub>*

0.082 cc. of cells in 3 cc. of  $0.5 \times 10^{-3}$  M KHCO<sub>3</sub> Temperature 25° Gas phase N<sub>2</sub> 4 per cent CO<sub>2</sub>. Vessels 2 and 4 have 0.2 gm. palladium black in side arm

		1	2	3	4
Volume of vessels, cc.		18.0	18.2	18.6	18.4
Volume of liquid, cc		3.2	3.2	3.3	3.5
K <sub>2</sub> CO <sub>3</sub>		1.60	1.63	1.65	1.63
KH <sub>2</sub>		1.46	1.38	1.37	1.37
Side arm		—	Pd	—	Pd
Dinitrophenol		—	—	0.6 × 10 <sup>-4</sup> M dinitrophenol	0.6 × 10 <sup>-4</sup> M dinitrophenol
	Observation period	Rates of pressure changes in $\frac{\text{mm.}}{30 \text{ min.}}$			
Dark	150 min.	+3	+4	+18	+18
Light	50 min.	-1.5	-3	+32	+18
		Gas exchange in 30 min. computed from above data			
Dark reaction		+6.5 c.mm. CO <sub>2</sub> +1.4 c.mm. H <sub>2</sub>		+30 c.mm. CO <sub>2</sub> ± 0 c.mm. H <sub>2</sub>	
Light reaction (corrected for dark reaction)		-11 c.mm. CO <sub>2</sub> +0.7 c.mm. H <sub>2</sub>		± 0 c.mm. CO <sub>2</sub> +19 c.mm. H <sub>2</sub>	
		Formation of free CO <sub>2</sub> and of acid in the course of 6 hrs.			
(a) Total CO <sub>2</sub>		+113 c.mm		+323 c.mm.	
(b) Loss of bicarbonate		40 c.mm. acid		253 c.mm. acid	
(a) - (b)		73 c.mm. free CO <sub>2</sub>		70 c.mm. free CO <sub>2</sub>	
(c) Lactic acid (expressed in c.mm of gas equivalents)		13 to 15 c.mm lactic acid		85 to 112 c.mm. lactic acid	

planation why in Table XVII the photochemical production of hydrogen in absence of carbon dioxide appears to be stimulated by dinitrophenol. The fermenting cell has continually some carbon dioxide at its disposal, in spite of the absorbing potassium hydroxide in the side arm of the vessel. These small amounts react with part of the hydrogen which becomes available for reduction by irradiation. Dinitrophenol may inhibit this reaction thus allowing more hydrogen to be developed.

Needless to say that very high concentrations of poison will tend to affect also the light reaction. At such high concentrations so many substances otherwise not toxic will interfere with the metabolism of plants that, generally, one cannot speak any more of the specific poisoning of a certain reaction.

#### DISCUSSION AND CONCLUSIONS

The occurrence of a hydrogen fermentation in some species of green algae is not surprising, once it has been shown that these species can utilize molecular hydrogen in light and dark reactions (1). It is known that hydrogenase systems in bacteria may work both ways and catalyze (as should be the case with a true catalyst) the uptake as well as the evolution of molecular hydrogen. Farkas and Farkas (33) have demonstrated that one function of a hydrogenase consists in establishing an equilibrium between molecular and bound atomic hydrogen. Without any metabolic process going on, preparations of resting *B. coli* caused the complete exchange of some deuterium gas against an equivalent amount of hydrogen that was present in abundance as water in the liquid phase. This agrees with our findings that in the dark the same algae release hydrogen from internal hydrogen donors in an atmosphere of nitrogen while they take up hydrogen in an atmosphere of hydrogen. Gaffron pointed out that the uptake of carbon dioxide without a corresponding production of oxygen by illuminated algal cells during the induction period after incubation in nitrogen should be regarded as a reaction with internal hydrogen donors quite similar to the photoreduction with free hydrogen. The inhibition of the reduction with hydrogen by added glucose or yeast extract (1) was explained accordingly not as an inhibiting but as a competitive effect. Our results not only support this view but suggest that, as far as the reduction of carbon dioxide in the light is concerned, there cannot be any distinction between the hydrogen originating from molecular hydrogen or from internal hydrogen donors, since the latter are in equilibrium with the former. Another question is that of the pathway of the reducing hydrogen from the donor to the photochemical mechanism. In the case of internal hydrogen donors, the hydrogenase is possibly not included in the mechanism of photoreduction. A comparison of reaction velocities and studies with algae like *Chlorella* which lack the hydrogenase system might decide this question.

So far no essentially new problems have arisen from the experiments de-

scribed in Part I of this paper. The situation is different, however, if we turn to the photochemical evolution of hydrogen in Parts II and III. This reaction is not only new but unique. No analogy to it has been reported even among the many reactions of the purple bacteria, the place where it might have been found. The essential characteristics of the new reaction are as follows:

1. It is not an acceleration of a dark fermentation.
2. Its magnitude depends on the amount of internal hydrogen donors.
3. It appears in place of a carbon dioxide reduction whenever this reduction is inhibited either by actual absence of the substrate or by poisoning.
4. It shares with fermentation, photoreduction, oxyhydrogen reaction, etc., of these algae the property that it cannot be observed under aerobic conditions or under anaerobic ones when the proper reduction of the essential enzyme system has been blocked by oxygen or poisons.

To the question about the origin of the hydrogen produced photochemically, one may give at present more than one answer. One may, for instance, assume a direct photochemical decomposition of hydrogen donors, a kind of a "photofermentation" as it were. We prefer, however, the following more adequate hypothesis. We assume that the hydrogen originates from water in the photochemical reaction, that it is part of the hydrogen destined to reduce carbon dioxide. Unable to reach the substrate, the hydrogen is transferred to the hydrogenase and there transformed into the free gas. The dependency of this reaction upon the presence of suitable hydrogen donors in the cell becomes understandable if we consider that the photochemical production of reducing substances from water demands an equivalent formation of oxidized counterparts. In case the reducing substances are not used in the reaction with carbon dioxide they will most likely form water again with those oxidized partners. Only inasmuch as the latter are reduced and taken care of by other reducing compounds (hydrogen donors) in the cell there is an opportunity for hydrogen made available by the photochemical reaction to be released as free gas by way of the hydrogenase system.

#### SUMMARY

1. After 2 hours of fermentation in nitrogen the metabolism of those algae which were found capable of photoreduction with hydrogen changes in such a way that molecular hydrogen is released from the cell in addition to carbon dioxide.

2. The amount of hydrogen formed anaerobically in the dark depends on the amount of some unknown reserve substance in the cell. More hydrogen is formed in presence of added glucose but no proportionality has been found between the amount of substrate added and that of hydrogen formed. This is probably due to the fact that two types of fermentation reactions exist with little or no connection between them. Whereas mainly unknown organic



acids are formed during the autofermentation, the addition of glucose causes a considerable increase in the production of lactic acid

3 Algae which have been fermenting for several hours in the dark produce upon illumination free hydrogen at several times the rate observed in the dark, provided carbon dioxide is absent

4 Certain concentrations of dinitrophenol strongly inhibit the evolution of hydrogen in the dark. Fermentation then continues mainly as a reaction leading to lactic acid. In such poisoned algae the photochemical liberation of hydrogen still continues

5 If the algae are poisoned with dinitrophenol the presence of carbon dioxide will not interfere with the photochemical evolution of hydrogen

6 The amount of hydrogen released in this new photochemical reaction depends on the presence of an unknown hydrogen donor in the cell, it can be increased by the addition of glucose but not in proportion to the amount added

7 The results obtained allow for a more correct explanation of the anaerobic induction period previously described for *Scenedesmus* and similar algae. The possibility of a photochemical evolution of hydrogen had not been taken into account in the earlier experiments

8 The origin of the hydrogen released under the influence of light is discussed

# REDUCTION OF CARBON DIOXIDE COUPLED WITH THE OXYHYDROGEN REACTION IN ALGAE

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Several species of algae have the capacity to include molecular hydrogen in their metabolism. This capacity becomes apparent only after anaerobic incubation and is lost again in contact with oxygen. While traces of oxygen are sufficient to prevent the appearance of the reactions with hydrogen, measurable amounts of oxygen are tolerated *after* adaptation to the hydrogen metabolism. This tolerance is due to the fact that the oxygen is rapidly reduced to water. The present investigation of this reduction, the oxyhydrogen reaction, in algae was undertaken when it was found that carbon dioxide is reduced in the dark simultaneously with the formation of water from oxygen. Though here observed for the first time in green plants this coupled oxidation-reduction of carbon dioxide is known to occur in several strains of bacteria. The surprisingly high yield of carbon reduced per molecule of oxygen in so called *Knallgas* bacteria has been pointed out by those who first discovered it (34) and gave rise to a treatise on thermodynamics in living cells (35). Since that time the problem has certainly not become less interesting. The question of how the formation of water is coupled with the reduction of carbon dioxide is still unsolved.

Now at a time when experiments with radioactively labeled carbon have shown that carbon dioxide can reenter along many ways the carbon cycle in living cells, this type of reaction deserves renewed attention. The dark reduction with hydrogen lies on the border line between autotrophic and heterotrophic carbon assimilation. Free hydrogen is the simplest of all reducing agents. Both autotrophic and heterotrophic bacteria are known in which molecular hydrogen can replace either inorganic or organic hydrogen donors. In the present case the dark reduction of carbon dioxide acquires a particular importance since it occurs in the same cell that carries on photoreduction and photosynthesis. Observations concerning the dark reduction could supplement our knowledge of the mechanism of photosynthesis. The investigations reported below have, indeed, revealed many striking similarities between light and dark reactions especially a certain parallelism in the effect of specific poisons.

Summarizing these results one can hardly avoid the conclusion that with the exception of the typical light absorption by chlorophyll both photoreduction and dark reduction of carbon dioxide in green algae proceed along the same

pathways. It would be premature, of course, to say that the bacterial dark reduction of carbon dioxide with hydrogen requires a similar mechanism.

### *Material and Method*

(a) *General Remarks*—Unicellular algae of the genera *Scenedesmus*, *Rhaphidium*, *Ankistrodesmus*, and perhaps many more, which contain or develop a hydrogenase system, can reduce oxygen with hydrogen under the proper anaerobic conditions. Several species of *Scenedesmus* have been used in most of the experiments, because they are easier to cultivate than the other algae named above. For culture methods see a recent review (36). The anaerobic treatment is the same as described for photoreduction (Papers I and II).

Warburg's manometric method has been used to measure the gas exchange of the algae, as in the previous investigations. The measurements of the oxyhydrogen reaction are in some respects simpler, in other respects more difficult than the experiments on photoreduction. They are simpler because there is no doubt that the complete reduction of oxygen with hydrogen yields water. They are more difficult because the gas exchange does not include two, but three gases:  $O_2$ ,  $H_2$ , and  $CO_2$ . While the nature of the reactions could be established beyond doubt, the determination of the number of molecules of carbon dioxide reduced per molecule of oxygen required the comparison of a large number of experiments since the result of a single measurement is liable to large fluctuations. The observed deviations, however, are not due solely to the method. Under anaerobic conditions in nitrogen, the algae ferment, producing carbon dioxide, organic acids, and hydrogen. If the fermentation is carried on under hydrogen, as was the case in the present experiments, the algae not only ferment but continue to absorb various amounts of hydrogen. (Compare Table XI in Paper II.) It is the underlying basal metabolism which often introduces an error up to 20 per cent in the individual measurement. This error lies always in the same direction. More hydrogen is absorbed during the oxyhydrogen reaction than is due solely to the latter. A proper correction is easily applied by deducting metabolic rates observed in control experiments or during the periods before and after the reaction in question.

(b) *Details of Method*—To prove the reduction of carbon dioxide in the course of the oxyhydrogen reaction the difference in the amount of total  $CO_2$  before and after the reaction has been determined. The initial amount was known either because an analyzed gas mixture was used to fill the manometer vessel, or because carbon dioxide was released previous to the measurement inside the vessel from a carbonate solution and could be measured manometrically. After the oxyhydrogen reaction had run to completion the remaining carbon dioxide was absorbed completely by a potassium hydroxide solution. Flasks serving a similar purpose, the so called Dixon-Keilin vessels, are described in the literature (37). The latter have a rather massive stopcock at the bottom of the flask which prevents their use in photochemical experiments. We have employed two other ways to absorb carbon dioxide. In the first method the side arm of the manometer vessel has a wide opening which is closed by a ground-in stopper protruding into the side arm in form of a paddle. By turning the stopper the paddle breaks a delicate glass bulb containing the potassium hydroxide solution. The solution, spreading over a piece of filter paper, now rapidly absorbs all carbon dioxide

(see Fig 14 b) No gas bubbles are allowed in the glass bulb. If it is important to know the gas volume and the amount of liquid in the vessel the glass bulb is weighed before and after filling. For filling it is simpler to boil some water in the bulb and dip the capillary end into the potassium hydroxide solution than to use vacuum in a desiccator. After breaking off most of the capillary the opening is sealed with a little paraffin or wax. A drawback of the method is that it depends so much on the skill of blowing the glass bulbs. A bulb which does not break when the paddle is turned means the loss of an experiment which has already been running for a day or two.

The second method depends on a type of vessel we have been using for quite some time. The side arm is connected with the main part of the vessel through a large hole in the ground joint where vessel and manometer fit together. The side arm can be closed and opened by turning the vessel around its vertical axis (Fig 14 a). The supporting springs are attached to a concentric ring turning freely around the capillary. It is unnecessary to remove the vessels from the thermostat or to touch them at all.

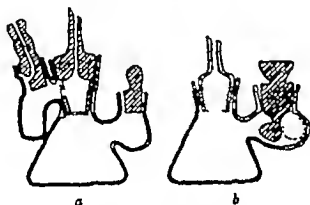


FIG 14 For explanation see text.

with the fingers. Thermal pressure changes are thereby prevented. The great advantage of using these vessels is that the side arm can be closed and opened several times in the course of an experiment. A disadvantage is the existence of a separate gas space above the potassium hydroxide solution. While the side arm remains closed the pressure changes in the main part of the vessel due to the algal metabolism plus the change in barometric pressure have no effect on the isolated gas space. When the side arm is opened again there will be a sudden equalization of pressure between the main and the side compartment. Two ways are open to take care of the difficulty. One way is to reset the manometer reading and to re-establish the pressure prevailing at the moment of the closing just before the side arm is reopened. The other requires the computation of the expected change in manometer reading. In any case it is recommendable to open the side arm first for a moment only to the point where a pressure exchange but no appreciable gas exchange occurs. This allows checking both methods as to the absence of any pressure jumps or as to the amount of the computed correction.

When a certain gas exchange occurs while the side arm is open the manometric reading will be different (smaller) than when the side arm is closed. In other words, we have two vessel constants,  $K_1$  and  $K_2$  for the smaller and for the larger gas volume

A certain gas exchange of  $\tau$  cubic millimeters then is given by

$\tau = P_1 K_1 = P_2 K_2$ , where  $P_1$  or  $P_2$  are manometric pressure changes in millimeters of Brodie solution (1,000 mm = 1 atmosphere = 762 mm Hg). When the gas exchange has taken place with the side arm closed and afterwards the arm is opened the reading  $P_1$  will in this moment change to  $P_2$ , just as if the gas exchange had occurred in the greater volume. The difference,  $\Delta P = P_1 - P_2$  is the correction which has to be applied after all the carbon dioxide has been absorbed. Since a continuous gas absorption begins once the side arm has been opened,  $\Delta P$  cannot be measured accurately. The difference can be computed according to

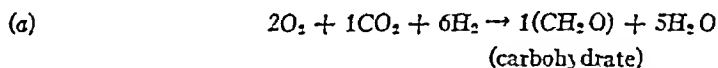
$$\Delta P = P_1 - P_1 \frac{K_1}{K_2} = P_1 \left( 1 - \frac{K_1}{K_2} \right) \quad (1)$$

$K_1$  and  $K_2$ , are the vessel constants according to Warburg's well known equations (See Dixon (37))

The solubilities of oxygen and hydrogen in water are small and approximately equal

$$\alpha_{O_2}^{25^\circ} = 0.03, \quad \alpha_{H_2}^{25^\circ} = 0.02$$

We can use, therefore, the same constants for computations involving the exchange of these two gases. Furthermore, since the vessel constants for oxygen and hydrogen are equal a plot of experimental data in millimeters of manometric readings will give an undistorted picture of the event as far as the oxyhydrogen reaction is concerned. The question arises whether it is allowed to proceed in this manner, if carbon dioxide is reduced together with oxygen. The following calculation shows that the error introduced by plotting all types of experiments in millimeters of gas pressure changes is slight. Let us assume that one-half molecule of carbon dioxide is reduced to the level of carbohydrate together with 1 molecule of oxygen. The balance of the reaction can be written



Only one-ninth of the disappearing gas volume is carbon dioxide. On account of its greater solubility in water the vessel constant for  $CO_2$  may be 1.6, for instance, instead of 1.3 as for the other gases.

In letting the reaction proceed with 50 c.mm. of oxygen we expect according to equation (a) at best a total gas uptake of 225 c.mm. This would correspond to a manometric pressure change of  $\frac{200}{1.3} + \frac{25}{1.6} = 170$  mm. Neglecting the different vessel constants for carbon dioxide and multiplying 170 with 1.3 we obtain 221 c.mm. instead of 225 c.mm. This is an error of 2 per cent.

It is very easy to introduce into several manometer vessels approximately the same amount of oxygen and to measure the amount introduced into each vessel very accurately, whereas one would need an elaborate apparatus to introduce exactly the same volume of oxygen into every one of the vessels. In sets of several parallel measurements the procedure was as follows. All manometers are set so that the pressure within the vessel is 300 mm. below atmospheric pressure (1 atmosphere =

10,000 mm Brodie solution) A vent stopper leading to the air is turned once very quickly. Some air is drawn into the vessel and no gas within the vessel can escape by diffusion against the air current. The manometer vessels are left standing for 3 to 5 minutes. The air introduced diffuses throughout the vessel and attains the temperature of the thermostat. Now a reading is taken and the vessels are set in motion. With the beginning of shaking the oxygen diffuses rapidly into the cell suspension and the reaction starts. A pressure difference of about 200 mm. before and after the introduction of air can be measured accurately. Since 0.207 is the percentage of oxygen in air, 200 mm. of air are equivalent to 41.4 mm. of  $O_2$ . The correct zero point for the beginning of the oxyhydrogen reaction is not the second reading taken while the vessels were still standing because the air has not reached the equilibrium distribution yet between the gaseous and the liquid phase. Consequently a little more gas than is due to the metabolic reaction will be absorbed by the cell suspension during the first minutes of shaking. Correction for this may be made by taking the solubility of air in the suspension medium into account. When several vessels are treated in the same way the amounts of oxygen introduced into the vessels differ from a few per cent up to 20 per cent.

The shape of the reaction curve is independent of the partial pressure of oxygen the rates are proportional to it. Consequently for comparison all curves can be superimposed. The data of one experiment are multiplied with a factor given by the ratio of the two different oxygen partial pressures. In Table XX the data of several measurements have been computed on the assumption that exactly the same amount of oxygen equal to 0.5 volume per cent had been introduced into the vessel. The obvious coincidence justifies the procedure outlined. All comparative measurements have been plotted in a like manner.

### *Influence of Carbon Dioxide on the Oxyhydrogen Reaction*

The simple reduction of oxygen with hydrogen to water by living cells should require an uptake of the respective gases in the ratio of  $1 O_2$  to  $2 H_2$ . If one introduces into a vessel containing a suspension of adapted algae an amount of oxygen corresponding to a rise of 50 mm. in partial pressure, one can expect the oxygen to disappear together with twice the volume of hydrogen. At the end of the reaction the manometric pressure should be 100 mm. lower than at the beginning of the experiment, before the addition of oxygen. Actually this is observed only under special conditions as we shall see below. An experiment was performed with hydrogen containing carbon dioxide of the same partial pressure as used in cultivating the algae. Another vessel contained pure hydrogen and potassium hydroxide solution in the side arm in order to absorb carbon dioxide which might be formed if some of the oxygen would not react with hydrogen but oxidize organic substances. Curves *a* and *b* in Fig. 15 are the observed results in presence or absence of carbon dioxide. Table XX contains similar experiments in more detail. Much more than the theoretical amount of gas is taken up by the algae in presence of carbon dioxide and less in its absence. The incomplete reduction of oxygen

TABLE XX

*Oxyhydrogen Reaction in Presence and Absence of Carbon Dioxide*

0.02 cc of cells of *Scenedesmus obliquus* species Pringsheim in 2 cc of 0.033 M phosphate buffer pH 5.4 (a) 0.2 cc of 0.1 M  $K_2CO_3$  added from a side arm after incubation. Vessel contains now 450 c mm of  $CO_2$ , 60 per cent of which are retained in the solution (b) 0.2 cc of 8 per cent NaOH in side arm to absorb all  $CO_2$ . Temperature  $25^\circ$ . Incubation 12 hours in  $H_2$ .

(a) $CO_2$ present							(b) $CO_2$ absent					
Volume of vessels, cc	14.9	16.0	15.0				15.4	15.2	15.4			
$O_2$ introduced, mm	57	46	60				41	48	44			
Time  min	Mm of gas absorbed, (1) observed, (2) calculated for 50 mm of $O_2$											
	1	2	1	2	1	2	1	2	1	2	1	2
60	76	67	66	72	87	72	33	40	36	37	33	37
120	144	126	119	129	162	135	59	72	67	70	61	69
150	166	146	137	149	185	154	68	83	77	80	70	80
180	180	158	148	161	199	166	71	86	83	86	75	85
240	203	178	165	179	221	184	81	99	93	97	85	97
300	214	187	172	187	230	192	85	104	97	101	88	100
360	219	193	176	191	235	196	87	106	100	104	91	103
420	224	196	179	195	239	199	90	110	102	106	93	106
580	226	198	182	198	243	203	91	111	107	111	95	108

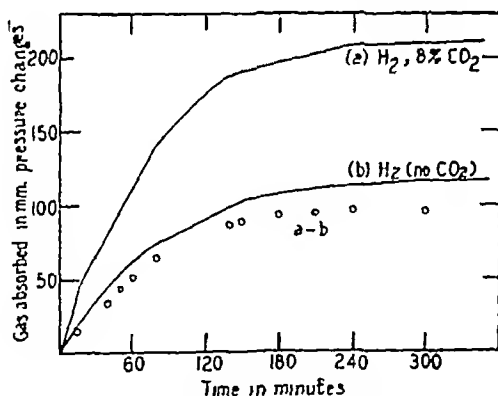


FIG 15 Oxyhydrogen reaction in presence and absence of carbon dioxide

is easily explainable by side reactions. The oxygen might partly be used up by hydrogen donors other than hydrogen or even by normal respiration. The excess absorption of hydrogen, however, indicates the simultaneous reduction of some substance other than  $O_2$ . Measurements of the total amount of

carbon dioxide before and after the completion of the oxyhydrogen reaction revealed that considerable amounts of carbon dioxide had disappeared together with the excess hydrogen. Table XXI contains some of the data. We see that the hydrogen taken up in excess over the amount necessary for

TABLE XXI

*Disappearance of Carbon Dioxide in the Course of the Oxyhydrogen Reaction in the Algae Scenedesmus and Rhabdium*

Ca. 0.03 cc. of cells in 3 cc. of various media Gas phase  $H_2$  with 0.5 to 1.7 vol. per cent  $CO_2$   $O_2$  introduced measured directly  $CO_2$  disappeared measured as difference between the initial and the final carbon dioxide content.  $H_2$  absorbed measured as difference between the total pressure change and the pressure change due to the absorption of oxygen and of carbon dioxide

Algae and medium	Duration of experiment	Gas exchange in experiment with oxygen			Gas exchange in fermenting control	
		$O_2$ introduced	$CO_2$ disappeared	$H_2$ absorbed	$CO_2$	$H_2$
	hrs	c.mm	c.mm	c.mm.	c.mm	c.mm.
<i>Scenedesmus D<sub>2</sub></i> (old culture) in M/15 phosphate buffer pH 6.5	44	278	90	609	—	—
<i>Scenedesmus D<sub>2</sub></i> in M/20 phosphate buffer pH 6.0	47	258	71	596	+2.5	+4
<i>Scenedesmus D<sub>2</sub></i> in culture medium	18	298	82	962	+34	-49
	12	267	75	761	+55	-105
	27	470*	130	820	+11	-8
<i>Rhabdium polymorphum</i> in culture medium pH 5.7	23	196	81	560	±0	-50
<i>Scenedesmus D<sub>2</sub></i> in culture medium	19	420	128	1 210	+11	-18
<i>Scenedesmus D<sub>2</sub></i> in M/20 $KH_2PO_4$	17	206	65	567	+5	-1

Not all oxygen absorbed. Algae turned to aerobic conditions, oxyhydrogen reaction stopped

the formation of water is not always sufficient to bring the carbon of the missing carbonic acid to the carbohydrate level. It can hardly be expected however that the ratio  $\frac{\text{Additional } H_2 \text{ absorbed}}{CO_2 \text{ disappeared}}$  will be exactly

2 in each experiment. The often apparently incomplete reduction of oxygen and the fermentation processes (see Paper II) are factors which must cause appreciable deviations. Since in photoreduction and photosynthesis the reduction of carbon dioxide proceeds to the level of carbohydrate, it is



very probable that also here carbohydrate is formed. As long as a chemical analysis of the substances produced under these conditions is wanting this remains, however, an assumption

*The Ratio of Carbon Reduced Per Molecule of Oxygen Absorbed*

In continuation of the experiments it was soon found that the course of the oxyhydrogen reaction, and with it the yield of reduced carbon, varied much more with experimental conditions than did photosynthesis in the same set of algae. A statistical survey of the results of 129 measurements gives a clear answer as to the maximum amount of gas absorbed per unit of oxygen

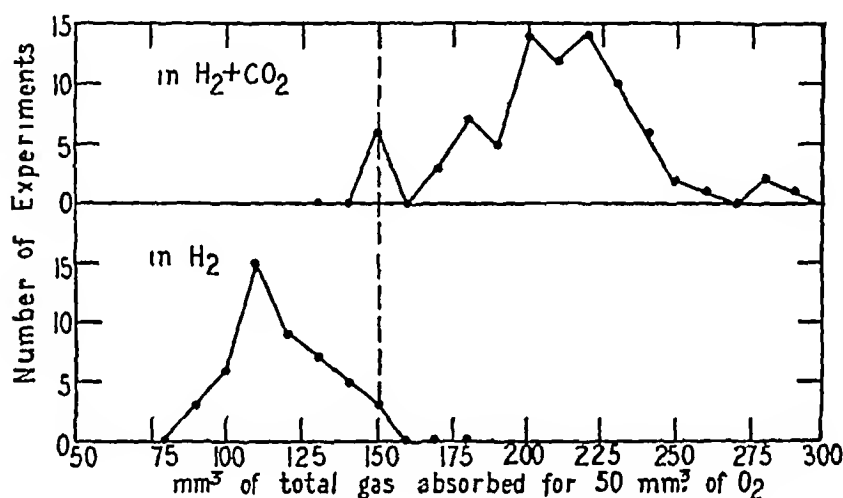


FIG 16 Total amount of gas absorbed upon addition of 50 c mm of oxygen in presence and absence of carbon dioxide

In Fig 16, the individual results have been arranged into columns 10 c mm apart. Data between the numbers 96 and 105, for instance, have been counted as 100, between 106 and 115 as 110, etc. These data fall into two groups, one in which carbon dioxide was known to be present and in another in which it was known or supposed to be absent.

In the group without carbon dioxide, the majority of experiments point to the absorption of only two volumes of gas ( $1 \text{ O}_2 + 1 \text{ H}_2$ ), the rest indicate an approach to a maximum of three volumes, ( $1 \text{ O}_2 + 2 \text{ H}_2$ ). In the other group, three volumes constitute the minimum yield found only in a fraction of all the experiments, while the majority shows an absorption of four to four and one-half volumes, that is, 200 to 225 c mm of gas for 50 c mm of oxygen. There is no doubt that the formation of water, *i.e.* the absorption of one part

of oxygen with two parts of hydrogen, is the exception. The oxyhydrogen reaction either stops before or surpasses it by including the reduction of some carbon dioxide. Therefore it is the presence of carbon dioxide which decides which course will be taken. We can state that as soon as carbon dioxide is present, the oxyhydrogen reaction goes to completion. Water is formed always, simultaneously carbon dioxide is reduced in varying amounts.

If we consider first the group of experiments where carbon dioxide was absent, we may be allowed to disregard those experiments in which water has

TABLE XXII

*Absence of Respiration during Oxyhydrogen Reaction*

0.03 cc. of cells of *Scenedesmus D<sub>2</sub>* washed with 0.03 M  $\text{Na}_2\text{HPO}_4$  then suspended in 3 cc. or 4.5 cc. of medium Gas phase  $\text{H}_2$  Temperature 25 Adaptation time 6 hours. Final  $\text{CO}_2$  content measured by acidifying with  $\text{H}_2\text{SO}_4$  Duration of experiment 2 days.

Volume of emuls, cc.	0.03 cc. of cells in 3 cc. 0.06 M $\text{Na}_2\text{HPO}_4$				0.045 cc. of cells in 4.5 cc. of phosphate buffer		
	pH 9.0				pH 7.9		
	16.7	18.2	18.4	18.5	15.0	16.0	15.3
Total amount of $\text{O}_2$ introduced in several portions of about 50 mm. each } mm	0	374	321	327	160	172	185
Total amount of gas absorbed } mm ( $\text{O}_2 + \text{H}_2$ )	(+7)	-651	-748	-776	-405	-464	-482
Difference of total $\text{CO}_2$ content of suspension before and after experiment } mm	+40	+24	+20	+25	$\pm 0$	-7	+14

apparently been formed and which overlap with similar results in the carbon dioxide group. Because fermentation continually supplies the cell with small amounts of carbon dioxide it is not surprising that part of the experiments in absence of known amounts of carbon dioxide should yield results as if this gas were present. Significant is the fact that in the absence of carbon dioxide the oxyhydrogen reaction fails to go to completion in most of the experiments. In these experiments the ratio of hydrogen to oxygen is about 1:1. We learn from these data that the two molecules of hydrogen which reduce oxygen to water are not equivalent in their mode of action. First an intermediate compound is formed from oxygen and one molecule of hydrogen, and it depends

on the presence of carbon dioxide whether the reaction will utilize more hydrogen to form water or continue in unknown directions. Carbon dioxide is not merely the hydrogen acceptor in a coupled reaction, but occupies in addition a key position directing the course of the oxyhydrogen reaction (Compare literature in reference 38.)

As to the fate of the intermediate compound in absence of carbon dioxide, it is unlikely that the substance accumulates. The incomplete reaction with oxygen can take place several times in succession without apparent damage to the algal cells (compare Table XXII). If such an intermediate (probably a kind of peroxide) would not disappear by a reaction with internal hydrogen donors, the first thing to happen would be the inactivation of the hydrogenase system. In fact, the latter invariably becomes inactivated, if the rate of oxygen uptake (determined by the partial pressure of oxygen) is too high or if the reaction is inhibited by cyanide (see below).

Turning now to the group of experiments in the presence of carbon dioxide in which the oxygen is completely reduced by hydrogen, we were interested to know how much gas would be absorbed in addition to the two volumes of hydrogen needed for the formation of water. From Fig 16, it is apparent that up to three additional volumes of gas absorbed all intermediate values may be found. Fig 16 shows that, after reaching a peak at about 225 c mm total gas absorbed for each 50 c mm of  $O_2$ , the number of experiments giving greater values drop off rapidly. No case is recorded where the total uptake surpasses 300 c mm of gas. An examination of the highest values above 250 c mm has shown invariably that they belong to experiments of very long duration or of large and questionable corrections.

50 c mm (or mm) of  $O_2$  is our "standard" volume. We conclude that not more than 1.5 volumes of additional gas are absorbed by the plant for 2 volumes of hydrogen utilized in the oxyhydrogen reaction. It has been shown (Table XXI) that carbon dioxide is taken up together with an excess of hydrogen. If in this reaction carbohydrate is produced, the ratio of carbon dioxide to hydrogen should be 1:2. With the understanding that the coupling between the formation of water and the reduction of carbon dioxide is stoichiometric, we can expect for each volume of oxygen absorbed the following maximal amounts of "extra" gas

- (1)  $\frac{1}{2} H_2 + \frac{1}{4} CO_2 = \frac{3}{4}$  volume
- (2)  $1 H_2 + 0.5 CO_2 = 1.5$  volumes
- (3)  $1.5 H_2 + 0.75 CO_2 = 2.25$  volumes
- (4)  $2 H_2 + 1 CO_2 = 3$  volumes

Of all possibilities only the second fits the great majority of observations and is further supported by other experiments reported below. There is no doubt that it is possible to obtain results which apparently agree with the third or

even fourth case, but only if the very end of the absorption process continuing for many hours is ascribed to the oxyhydrogen reaction and not to a secondary absorption of hydrogen connected with fermentation, and if no correction is applied for this continuing metabolism as well as for the gas exchange observable prior to the experiment. The observed yield of 0.5 molecule  $\text{CO}_2$  for 1 molecule  $\text{O}_2$  is the highest well established value in the literature on the dark reduction of carbon dioxide coupled with the oxyhydrogen reaction. Ruhland's (34) best results gave 0.3 molecule  $\text{CO}_2$ .

#### *The Part Played by Respiration during the Oxyhydrogen Reaction*

In all cases where the oxyhydrogen reaction proceeds with the absorption of two or more equivalents of hydrogen for each molecule of oxygen, there is little doubt that practically none of the oxygen absorbed by the cells is used in normal respiration. In discussing the fact that the incomplete reaction in absence of carbon dioxide, which leads to the uptake of one molecule of hydrogen only, is not harmful to the cell, it was concluded that the first intermediate must react further and disappear by way of an internal reduction. In connection with this unknown step, a formation of carbon dioxide appeared possible. Table XXII shows the results of several experiments made with the purpose of establishing any such production of carbon dioxide. The algae were suspended in alkaline phosphate solution which retains any carbon dioxide released from the cells as carbonate or bicarbonate. The partial pressure of free carbon dioxide was so low that the reaction remained incomplete, as explained in the preceding paragraph. Only about one molecule of hydrogen was taken up for each molecule of oxygen absorbed. The quantities of oxygen used were much greater than in most of the other experiments. To make this possible, small and tolerable amounts of oxygen were introduced repeatedly. The difference determined by acid titration between the carbonate content of the solution before and after the experiment was always less than one-tenth of the corresponding amount of oxygen. This small production of carbon dioxide can easily be accounted for by fermentation. The amount of carbon dioxide formed during the same time in a fermenting control was even larger. Since we can hardly assume that the first step in the oxyhydrogen reaction leads to a very stable intermediate, it follows that the internal hydrogen donor reacting in place of the second molecule of hydrogen does not yield carbon dioxide in this process. Certainly normal respiration is suppressed completely under the circumstances favoring the oxyhydrogen reaction. This is remarkable because less than 1 per cent of oxygen can still support respiration in these algae when the low partial pressure is applied in direct exchange for air. On the other hand, once a return to aerobic conditions has been enforced by a small 'excess' of oxygen the oxygen uptake now continues through respiration.

### *Partial Reactions*

In the preceding paragraph it has been shown that the course of the oxyhydrogen reaction involves two or more steps which differ in importance for the induced reduction of carbon dioxide. A study of these partial reactions is encouraged by the fact that they are clearly demonstrated when the course of the reaction is plotted in curves showing either the total amount of gas absorbed, or the rate of absorption, *versus* time. The same breaks or sudden changes in slope which can be seen in Figs 15, 19, 21, and 22 have been verified in 75 per cent of more than a hundred single experiments. Small deviations in the readings are sufficient to obliterate this peculiarity. No such well defined breaks are obtained if the curves are drawn through observation points which are few and far apart or if the data of several parallel experiments are averaged and plotted.

Our interpretation of the broken curves is that the very first partial reaction is faster than some of those which follow. In general the curves have three to four distinct breaks and hence consist of as many straight lines of different slope. In several cases these straight lines, when traced back to the ordinate, intersect it at points having the same number of millimeters or multiples thereof as the initial partial pressure of oxygen. This observation is general and therefore cannot be ascribed to chance. The obvious interpretation is that the absorption of oxygen, of hydrogen, and of carbon dioxide starts nearly simultaneously, each of these reactions proceeding at a different rate. Probably the rates of absorption for each one of the three hydrogen equivalents are also different. Whenever a partial reaction has come to completion, its rate drops to zero, and consequently the overall rate diminishes. The initial absorption of oxygen is artificially maintained at a slow rate. Since it sets the pace, any ensuing partial reaction faster than this one remains unnoticed. Two problems present themselves (1) Which partial reaction, if its rate becomes too fast, leads to inactivation? and (2) Which partial reaction is coupled with the reduction of carbon dioxide? In answer to the first question, we can state that the rate of oxygen absorption rises in proportion to the partial pressure of  $O_2$ . (Compare Table XX.) Depending on individual conditions of the algal cultures, up to two volumes per cent of oxygen may be tolerated. Inactivation is observed usually above one volume per cent of  $O_2$ . The maximum rate obtainable without inactivation is approximately equal to the maximum rate of photoreduction in the same cells. (Compare Table IV in reference 22.) With a small "excess" of oxygen inactivation is a comparatively slow process. Large amounts of oxygen (ten to twenty volumes per cent) produce momentarily a very high rate of oxygen uptake followed by quick inactivation. The very first uptake of oxygen, then, seems to be a fast reaction leading to the accumulation of an intermediate which, if it is not removed, brings about inactivation. This agrees with the observation that

more oxygen, than would be tolerated initially, may be added to the cell suspension after the first break in the velocity curve has been passed. It is therefore possible to maintain the reaction for an indefinite period of time at the initial or even at a higher rate. Consequently oxygen will be consumed by the cells in large quantities. The experiments of Table XXI, proving the disappearance of carbon dioxide, as well as those of Table XXII, were made in this manner.

As to the coupling with the carbon dioxide reduction it is significant that from the start of the oxygen absorption the rates in the presence or absence of this gas differ. If the reduction of carbon dioxide were a slow reaction the experimental observation presumably would have been a prolonged uptake of hydrogen at a more or less steady rate in excess of the amount necessary to form water. Fig. 15 shows that this is not the case. The initial rate in presence of  $\text{CO}_2$  is approximately doubled. Plots of the difference of the curves with and without carbon dioxide demonstrate that the induced reduction is completed in about half the time required for the total process (Figs. 20 and 21).

### *Influence of Cyanide*

The inhibition of adaptation to the hydrogen metabolism by cyanide has been described in Paper I. Here we shall discuss the manner in which cyanide influences the oxyhydrogen reaction after adaptation has been completed. Table XXIII shows that relatively small concentrations of cyanide inhibit the oxyhydrogen reaction definitely. If larger amounts of cyanide are added in advance hardly any hydrogen is absorbed, the oxygen disappears slowly, and the algae lose the ability of performing any kind of metabolism. Hence the effect of very high concentrations of cyanide hardly permits one to draw conclusions as to the mode of action of the poison (contrary to the experiments with high concentrations of hydroxylamine). The experiment shown in Fig. 17 was made with a cyanide concentration which inhibited photoreduction about 50 per cent. The coupled reduction of carbon dioxide is stopped whenever HCN is added. The reduction of oxygen continues. A comparison of the action of cyanide in the presence (Fig. 17) or absence (Fig. 18) of carbon dioxide clearly demonstrates that it is the induced reduction of carbon dioxide which is most sensitive to cyanide. The first part of the oxyhydrogen reaction leading to a "peroxide" takes place. (The word "peroxide" here is used only as a short expression for the fact that oxygen and hydrogen are taken up in equal volumes.) From then on a further reduction of the "peroxide" is inhibited. If "peroxide" is formed and accumulates it would not be surprising to observe that the hydrogenase system becomes inactivated whenever the oxyhydrogen reaction occurs in the presence of cyanide. The percentage of inactivation is related to the time at which the poison is added after the start of the oxyhydrogen reaction. (Compare Table XXIV.) These findings

agree well with the observation that cyanide enhances the return of photoreduction to normal photosynthesis and that it prevents the adaptation even at concentrations which, added after adaptation, permit photoreduction to proceed at a measurable rate. In the experiment of Table XXIII, for instance, where during the oxyhydrogen reaction the hydrogenase system becomes inactivated in the presence of cyanide, we find only 13 per cent inhibition of photoreduction before and a normal rate of photosynthesis after the turnback

TABLE XXIII

*Inactivation of the Hydrogenase System by Oxygen in Presence of Cyanide*

0.048 cc of cells of *Scenedesmus D<sub>3</sub>* in 4 cc of 0.05 M phosphate buffer pH 6.5 Gas phase H<sub>2</sub>, 8 per cent CO<sub>2</sub> Temperature 26° Poison added anaerobically 2 hours before experiment

		1	2
Volume of vessels, cc		16.0	15.4
Cyanide concentration		—	$2 \times 10^{-4}$ M HCN
	Observation time	Rate of gas exchange in $\frac{\text{mm}}{10 \text{ min}}$	
(a) Light 2000 lux Photoreduction	17 min	-52	-45
(b) Dark Oxyhydrogen reaction with 50 mm of O <sub>2</sub>	After 10 min	-22	-8
	After 60 min	-15	-5
	After 120 min	-10	-1
(c) Light 2000 lux Photoreduction in (1) Photosynthesis in (2)	20 min	-66	+9

*Influence of Hydroxylamine*

With hydroxylamine the effects appear to be very complex. Firstly This poison, contrary to cyanide, may be added (after adaptation of the algae) in large concentrations before or during the oxyhydrogen reaction with hardly any effect upon its course. One obtains results similar to those shown in Fig 19, which should be compared with Fig 17. If, however, the presence of poison is tested by irradiation of the plant, one observes that photoreduction is inhibited, the turn more difficult, and any possibility for oxygen production completely blocked. The poison must have penetrated into the cell and into the chloroplast. See Table XXV, No 1, *d-h*.

Secondly Hydroxylamine, if added anaerobically as in the first case, is often found to inhibit strongly the coupled reduction of carbon dioxide. This is demonstrated in Figs 20 *a* and *b* showing the course of the oxyhydrogen

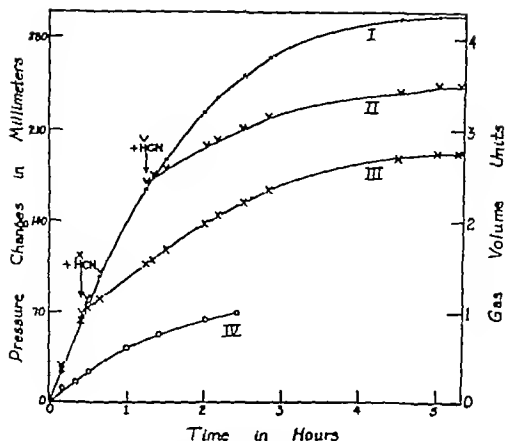


FIG. 17 Inhibition of the dark reduction of carbon dioxide by  $2 \times 10^{-4}$  M cyanide. Poison added at different times in the course of the oxyhydrogen reaction. The gas phase in this experiment was  $H_2$  with 4 per cent  $CO_2$ .

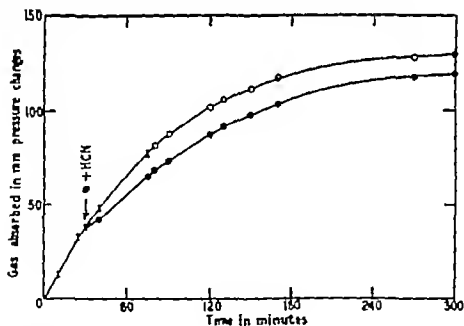


FIG. 18 Effect of  $2 \times 10^{-4}$  M cyanide upon the oxyhydrogen reaction in absence of carbon dioxide

reaction with and without poison. The absorption of oxygen continues in the poisoned algae together with either one or two equivalents of hydrogen



Curve *c*, the difference between curve *a* and *b*, demonstrates the time course of the partial reaction concerned with the reduction of carbon dioxide. In order to stop the dark reduction of carbon dioxide, the amount of hydroxylamine has to be at least as great as required for a measurable inhibition of photo-reduction, that is much larger than needed for a complete inhibition of photosynthesis. When hydroxylamine is added, however, aerobically before adaptation, the same reaction occurs that was described for photoreduction. The

TABLE XXIV

*Inactivation of the Hydrogen Metabolism by Small Amounts of Oxygen in Presence of Cyanide*

0.05 cc of cells of *S. obliquus* in 4 cc of 0.02 M  $\text{KH}_2\text{PO}_4$ . Temperature 25°. Gas phase  $\text{H}_2$ , 4 per cent  $\text{CO}_2$ . Preceding dark period 12 hours. Cyanide added at successive time intervals in different vessels during the course of the oxyhydrogen reaction. Final concentration of poison  $2 \times 10^{-4}$  M. Remaining activity tested by photoreduction after completion of the dark reaction.

Condition	Time	1	2	3	4
		Rate of pressure changes in $\frac{\text{mm}}{10 \text{ min}}$			
(a) Light 2600 lux	<sup>min</sup> After 10	-56	-62	-54	-66
(b) Dark Reaction with 70 mm $\text{O}_2$	After 30	-19	-21	-22	No $\text{O}_2$ added
		+ HCN		-17	
	75	-7.5	-20		
			+ HCN		
	150	-5	-4.5	-7.5	
	300	-1	-1	-1	
				+ HCN	
(c) Light 2600 lux	After 5	-10	-24	-34	-66
	10	+1	0	-28	-73
	15	+6	+6	-4	—

inhibition is either complete for lack of adaptation, or it is partial and then the oxyhydrogen reaction proceeds only to the intermediate "peroxide" level (Fig 21). See also Table XXV, Nos 2 and 3.

Thirdly. In some cases where the oxyhydrogen reaction yielded only water and for no apparent reason was not coupled with the reduction of carbon dioxide, addition of hydroxylamine restored the coupling. In other words, more hydrogen was absorbed in presence of the poison than without it. The experiment could be repeated several times and it is certainly not due to an error. See Fig 22.

Summarizing the experiments with hydroxylamine, one can say that the poison has no effect on the course of the oxyhydrogen reaction unless its con-

centration is unusually high, and that, if it takes effect, it will inhibit the reduction of carbon dioxide, whereas the reaction between hydrogen and oxygen may continue to the peroxide level or to the formation of water

The much greater inhibitions found when the hydroxylamine is added aerobically before the adaptation are a problem which does not concern the oxyhydrogen reactions as such. Photoreduction as well as the oxyhydrogen reaction can be used as tests for this peculiar type of inhibition.

We know now that the turnback from photoreduction to normal photosynthesis and to aerobic conditions under the influence of excess light is

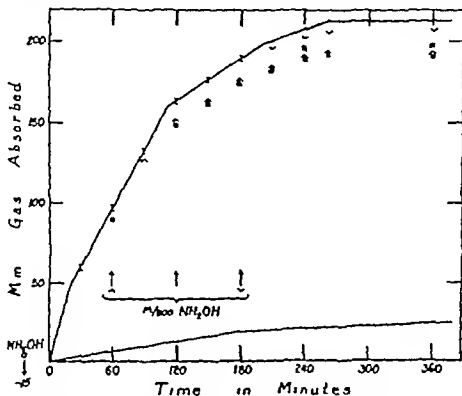


FIG 19 Effect of 1/800 hydroxylamine on the oxyhydrogen reaction ( $\text{CO}_2$  present) Poison added after adaptation.

prevented or retarded by hydroxylamine (Paper I). No such 'protection' by hydroxylamine exists if the turn is enforced by an excess of oxygen. This is the one point where photoreduction and oxyhydrogen reaction do not give corresponding results. On the other hand hydroxylamine does not enhance the inactivation as does cyanide.

One might think that the peculiar differences found in the effect of hydroxylamine were caused by the use of different strains of algae or of algal samples grown under unequal conditions. Table XXV proves that this is not the case. With the same culture distributed equally in four vessels, most of the reactions described were observed. In vessel 2, sufficient hydroxylamine was added aerobically to inhibit the rate of normal photosynthesis by 60 per cent. The intensity of illumination is so low that there is still proper

tionality between rate and intensity The inhibition remains the same however, when the intensity is cut in half This conforms to the observation of Weller and Franck (6) that hydroxylamine inhibits some photochemical

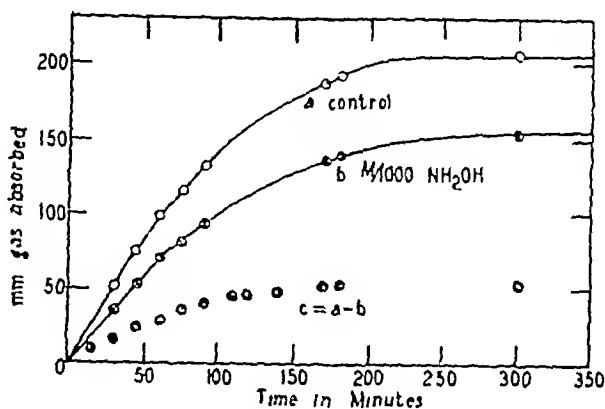


FIG 20a

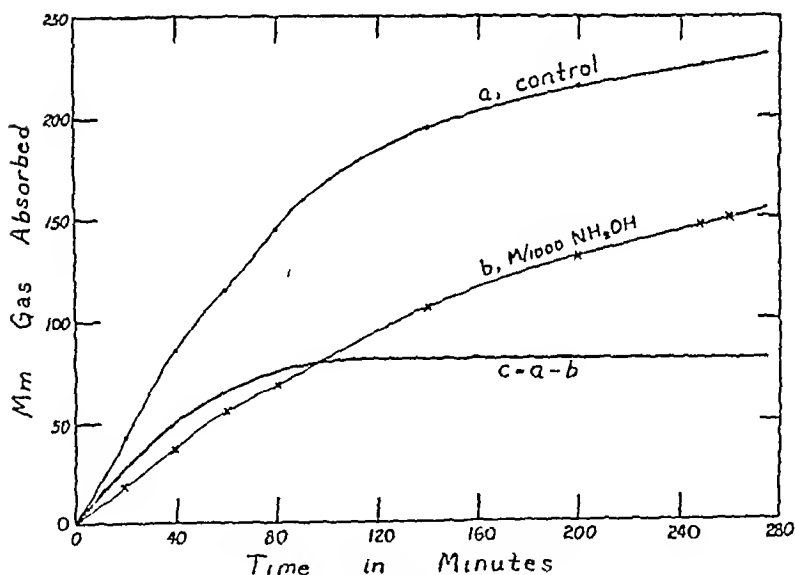


FIG 20b

FIGS 20a and 20b Inhibition of the dark reduction of carbon dioxide by hydroxylamine Poison added after adaptation

reactions in photosynthesis, in spite of the fact that it seems to inhibit specifically the production of oxygen (Paper I) During adaptation to hydrogen, the gas exchange of the poisoned algae is affected strongly, yet after 6 hours we find typical photoreduction at about 50 per cent of that of the unpoisoned algae This inhibition lasts for a dark period of about 20 hours During this time some oxygen has been added twice and the oxyhydrogen reaction proceeds

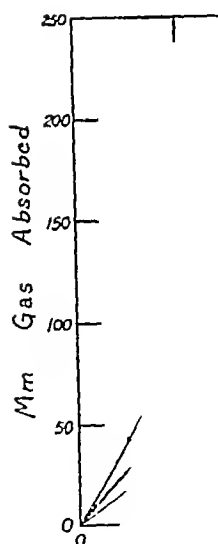
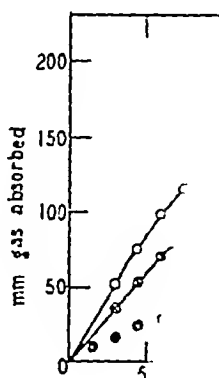
TABLE XXV

*Inhibition of Photoreduction and Dark Reduction by Hydroxylamine*

0.034 cc. of cells of *Scenedesmus D<sub>3</sub>* in 4 cc. of 0.1 M bicarbonate solution (equal parts of NaHCO<sub>3</sub> and KHCO<sub>3</sub>) Gas phase H<sub>2</sub> 4 per cent CO<sub>2</sub> Temperature 25° Alternate dark and light periods Light reactions measured as rates of gas exchange in mm./10 minutes. Dark reaction measured as millimeters of total gas absorbed for 50 mm. of O<sub>2</sub>.

No. of experiment		1	2	3	4
Volume of vessels cc		15.2	15.0	15.4	14.9
Poison added.		—	M/5000 NH <sub>2</sub> OH	—	—
Condition	Time	Photosynthetic rate in mm 10 min.	Photosynthetic rate in mm 10 min.	Photosynthetic rate in mm 10 min.	
Not adapted					
(a) Light. 3200 lux	5 min	+34	+14	+34	—
1700 lux	20 min	+20	+8	+19	—
(b) Dark, refilled with H <sub>2</sub> /CO <sub>2</sub>					
Adaptation	300 min.	-2.7	-1 → 0.5	-2.7	-3
		Photo-reduction in mm 10 min.	Oxy-reduction in total mm.	Photo-reduction in mm 10 min.	Oxy-reduction in total mm.
				+M/1000 NH <sub>2</sub> OH	
				Photo-reduction in mm 10 min.	Oxy-reduction in total mm.
(c) Light. 800 lux	10 min	-42	-24	-16	—
(d) Dark + 50 mm. O <sub>2</sub>	14 hrs.	250	195	151	—
Dark + 50 mm. O <sub>2</sub>	6 hrs.	207	188	109	—
(e) Light. 800 lux	30 min	-32	-24	-4	New control
(f) Dark	30 min.	+ M/1000 NH <sub>2</sub> OH	+ M/5000 NH <sub>2</sub> OH	—	—
(g) Light. 800 lux	15 min.	-36	-37	-7	-42
Light. 3200 lux	5 min.	-60	-78	-4	-78
Light. 3200 lux	10 min	-70	-102	-5	+4(f)
(h) Dark	2 hrs.	—	—	—	—
Dark + 50 mm. O <sub>2</sub>	5 hrs.	220	230	150	258
Dark + 50 mm. O <sub>2</sub>	16 hrs.	209	224	241	248
(i) Light. 800 lux	30 min.	-27	-32	-30	-39
Light. 3200 lux	20 min.	-47	-94	-78	-72 → +22(f)
(f) Dark + 50 mm. O <sub>2</sub>	3.5 hrs	211	225	208	209
(k) Light. 3200 lux	10 min.	-40	-103	-102	-88

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TABLE XXV

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No. of experiment		1	2	3	4
Volume of vessels cc		15.2	15.0	15.4	14.9
Poison added.		—	M/5000 NH <sub>2</sub> OH	—	—
Condition	Time	Photosynthe- sis rate in mm. 10 min.	Photosynthe- sis rate in mm. 10 min.	Photosynthe- sis rate in mm. 10 min.	
Not adapted					
(a) Light. 3200 lux	5 min.	+34	+14	+34	—
1700 lux	20 min.	+20	+8	+19	—
(b) Dark refilled with H <sub>2</sub> /CO <sub>2</sub> Adaptation	300 min	-2.7	-1 → 0.5	-2.7	-3
		Photo- reduc- tion in mm. 10 min.	Oxy- reduc- tion in total mm.	Photo- reduc- tion in mm. 10 min.	Oxy- reduc- tion in total mm.
		Photo- reduc- tion in mm. 10 min.	Oxy- reduc- tion in total mm.	Photo- reduc- tion in mm. 10 min.	Oxy- reduc- tion in total mm.
(c) Light. 800 lux	10 min	-42	-24	-16	—
(d) Dark + 50 mm. O <sub>2</sub>	14 hrs.	250	195	151	—
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(f) Dark	30 min.	+ M/1000 NH <sub>2</sub> OH	+ M/5000 NH <sub>2</sub> OH	—	—
(g) Light. 800 lux	15 min.	-36	-37	-7	-42
Light. 3200 lux	5 min.	-60	-78	-4	-78
Light. 3200 lux	10 min.	-70	-102	-5	+4(1)
(h) Dark	2 hrs.	—	—	—	—
Dark + 50 mm. O <sub>2</sub>	5 hrs.	220	230	150	258
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(i) Light. 800 lux	30 min.	-27	-32	-30	-39
Light. 3200 lux	20 min.	-47	-94	-78	-72 → +22(1)
(j) Dark + 50 mm.					

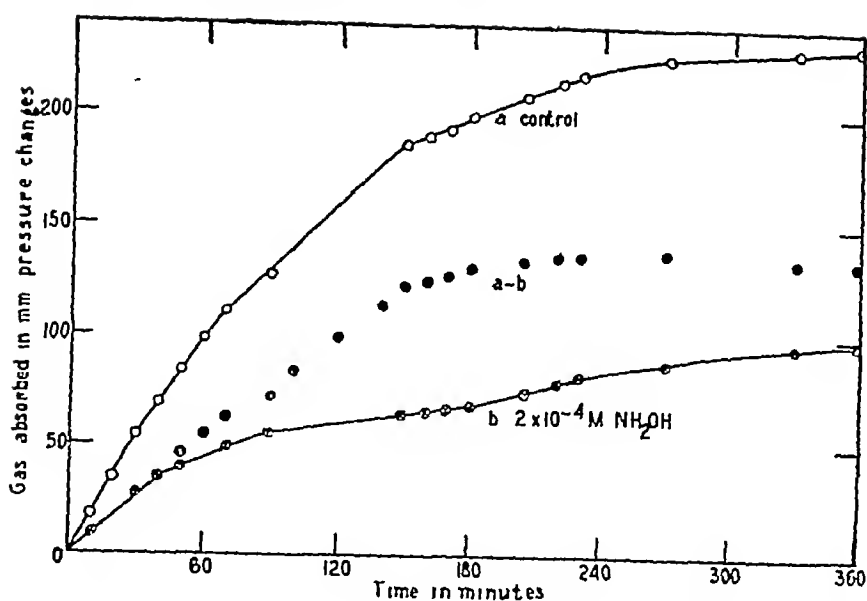


FIG 21 Inhibition of the dark reduction of  $\text{CO}_2$  by hydroxylamine Poison added in air before adaptation

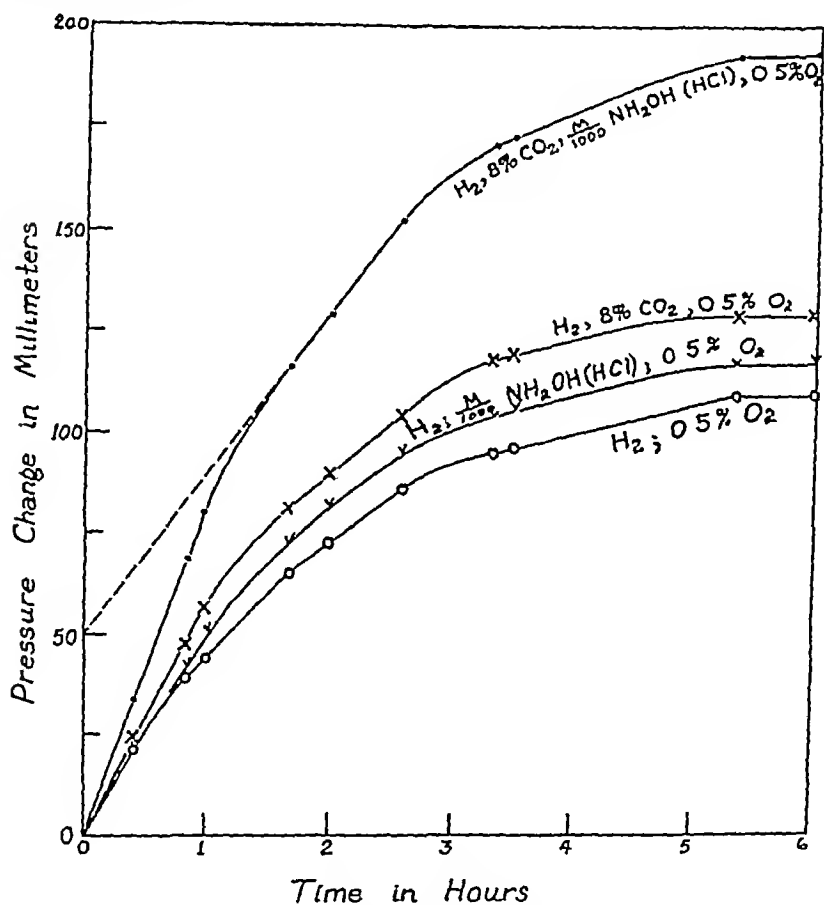


FIG 22 Effect of hydroxylamine upon the incomplete dark reduction of carbon dioxide

in a partially inhibited form. Adding now the same amount of poison which the algae had received aerobically the day before, not only produces no further inhibition, but it enhances suddenly the rate of photoreduction from 24 to 37 mm./10 min. and the value for the oxyhydrogen reaction attains the theoretical maximum. The presence of the poison is evident from the protection it provides against the turn to nerohic photosynthesis under the influence of the fourfold intensity. Compare vessel 4.

In vessel 3, hydroxylamine is added after 6 hours of adaptation. A concentration of  $10^{-3}M$  instantaneously produces a strong inhibition of photoreduction (60 per cent) and of the dark reduction. Both effects increased

TABLE XXVI

*Effect of Dinitrophenol on the Coupled Dark Reduction of Carbon Dioxide Compared with the Inhibition of Photoreduction*

0.027 cc. of cells (a) in 4 cc. of  $0.03 M KH_2PO_4$  (b) in  $0.05 M$  phosphate buffer pH 6.0  
Gas phase  $H_2$  4 per cent  $CO_2$

	(a)			(b)	
	Dinitrophenol added after 15 hrs. of adaptation Temperature 25°			Dinitrophenol added aerobically before adaptation Temperature 24°	
Final concentration of dinitrophenol	0	$2 \times 10^{-4} M$	$1 \times 10^{-3} M$	0	$2 \times 10^{-4} M$
Relative rates of photosynthesis at 3300 lux	100	100	50	100	55
Relative rates of photoreduction at 1700 lux	100	85	60	100	60
Oxyreduction. Total gas absorbed in mm. for 50 mm. of $O_2$	210	218	204	212	154

first with time and then vanish completely on the 3rd day. In the case of the dark reduction, the changes are not gradual but in clear-cut steps corresponding to the formation of water, then "peroxide" again water, and finally uninhibited coupled reduction.

The same amount of poison if added not after 6 but after 26 hours of anaerobic conditions leaves the yield of the oxyhydrogen reaction unchanged and inhibits photoreduction measurably only at higher light intensities.

The parallelism between the dark and the photoreduction of carbon dioxide with respect to the influence of hydroxylamine is striking. In general photoreduction remains more sensitive. No quantitative determinations have been made on the disappearance of the hydroxylamine from the algal suspension but it is certain that it decomposes slowly by way of side reactions in the course of an experiment lasting several days. Similarly a disappearance of the effect upon the oxyhydrogen reaction is accompanied by a fading of the



inhibition of photoreduction Nearly always enough poison remains in the solution to prevent normal photosynthesis under aerobic conditions

### *The Influence of Dimetrophenol*

Photosynthesis and photoreduction with hydrogen are inhibited strongly by dimetrophenol (Paper I) The oxyhydrogen reaction appears to be less sensi-

TABLE XXVII

#### *Inhibition of Hydrogen Absorption by Glucose*

0.034 cc of cells of *Scenedesmus D<sub>3</sub>* in 4 cc 0.1 M bicarbonate Temperature 25° Gas phase H<sub>2</sub>, 4 per cent CO<sub>2</sub> Cells 4 days in H<sub>2</sub>

	1	2	3
Volume of vessels, cc	15.4	15.0	14.9
Oxyhydrogen reaction Mm of total gas absorbed for 50 mm of O <sub>2</sub>	211	190	217
Poison added in (3) 20 hrs dark	—	—	+10 <sup>-3</sup> M NH <sub>2</sub> OH
Rate of photoreduction (mm / 5 min)	37	38	32
Glucose added, 30 min dark	—	+0.5 per cent glucose	+0.5 per cent glucose
Rate of photoreduction (mm / 5 min)			
First 5 min	28	15	6
After 15 min	39	34	22
Oxyhydrogen reaction * Mm of total gas (corrected) absorbed for 50 mm O <sub>2</sub> during 8 hrs	221	96	75
Rate of photoreduction (mm / 5 min)			
First 5 min	24	12	8
Second 5 min	36	30	20
After 14 hrs dark and 20 min light	35	37	16

\* Compare Fig 23

tive to this poison, as shown in Table XXVI If an inhibition occurs, then it is again the coupled reduction of carbon dioxide, not the formation of water, which is inhibited

### *The Influence of Glucose*

The photochemical utilization of molecular hydrogen is looked upon as the simplest case of a photoreduction which might as well proceed with the aid of other more complicated hydrogen donors (1, 22) One of the main points supporting this view is the fact that yeast extracts containing carbohydrates

or glucose greatly inhibit the uptake of hydrogen in photoreduction (1) and enhance the photochemical production of hydrogen in the absence of reducible carbon dioxide (Paper II). Glucose (in dilute solution) certainly has no inhibitory effect on respiration, fermentation, or photosynthesis in the same algae. A few experiments have been made to show that also with glucose there is parallelism between the induced dark reduction and photoreduction. In a medium containing glucose (0.05–1 per cent) the algae absorb less hydrogen in the course of the oxyhydrogen reaction than in the glucose-free control.

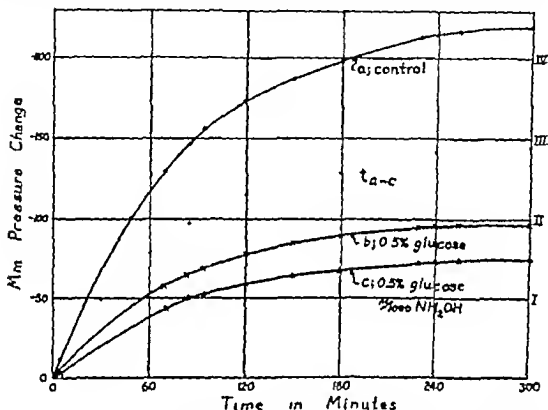


FIG. 23 Inhibition of the hydrogen uptake during the oxyhydrogen reaction in presence of glucose.

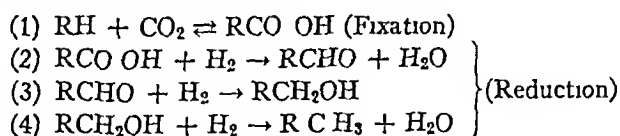
It is not surprising that the differences between the results obtained with and without glucose vary widely from experiment to experiment, since the activities of the competing enzyme systems need to change only little in order to produce greatly divergent results. The important problem is how much the uptake of hydrogen can be suppressed by the presence of glucose.

In the experiment shown in Table XXVII 0.5 per cent glucose did not greatly influence photoreduction before or after the reaction with hydrogen except during the first 5 minutes of illumination. The amount of gas absorbed in the course of the oxyhydrogen reaction, however, is less than one-half of the normal. In a second experiment a similar batch of algae in the same medium was used but it contained about 0.001  $\alpha$  hydroxylamine which had been added anaerobically a few hours earlier. These algae could be expected to absorb at least one or two equivalents of hydrogen. The curves in Fig. 23

demonstrate the suppression of hydrogen absorption. In presence of glucose only little more gas disappears than corresponds to the amount of oxygen introduced. The "excess" gas might be carbon dioxide in this particular case. Since the supply of oxygen is small compared with that of glucose it is probable that the latter is oxidized only partly and without a release of  $\text{CO}_2$ . Further experiments are necessary to prove definitely a reduction of carbon dioxide coupled with the partial oxidation of glucose. It has been mentioned before that the algae absorb considerable amounts of hydrogen during the first hours of anaerobic incubation. It appears likely that such partially oxidized carbohydrates play a part as acceptors for hydrogen.

#### DISCUSSION AND CONCLUSIONS

Lately many papers have been published concerning dark reactions in living cells which involve carbon dioxide as a reactant. (For a recent review on the subject see reference 38.) In comparing different results it is important to distinguish clearly what is meant by "carbon dioxide reduction." In the literature we find reduction, fixation, assimilation, etc., of carbon dioxide treated as if these words were synonyms. For instance, Ruben, Kamen, and Hassid (4), using the radioactive tracer method, have shown that, contrary to what has been found under the conditions described in this paper, carbon dioxide is not reduced in the dark under normal aerobic conditions. What they found is a reversible fixation of carbon dioxide in the plant which is a reaction postulated as an important preliminary step in photosynthesis since the times of Willstätter and Stoll. If we write this fixation, following the authors, as a carboxylation it is evident where the difference between the first equation and the following ones lies



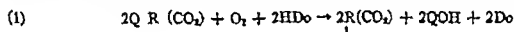
In the methane bacteria (25, 39) the carbon is completely reduced, but the methane is liberated from the cell, hence it cannot be assimilated. In liver cells (40) carbon dioxide is fixed and assimilated, but perhaps not reduced. In this and similar cases, it is not known whether after fixation in the form of a carboxyl group the carbon becomes reduced, and little attention has been paid thus far to the different possibilities. A clear terminology may help in clarifying the problems.

The data presented in this paper prove that in *Scenedesmus* and similar algae carbon dioxide is reduced by molecular hydrogen in the dark with the aid of the oxyhydrogen reaction. The reduction proceeds to the level of carbohydrate. The yield is one-half molecule of carbon dioxide reduced for one molecule of absorbed oxygen.

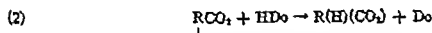
The problem confronting us now is to devise a mechanism which will explain how this yield is possible. We find in a paper of Yamagata and Nakamura a plausible scheme (41) for a pure oxyhydrogen reaction. A hydrogenase and an oxygen-transferring system will readily yield water, but nothing else.

In order to link the oxyhydrogen reaction with a reduction of carbon dioxide, we have to make use of the following experimental facts. (1) The presence of carbon dioxide is necessary for the undisturbed formation of water, otherwise the reaction proceeds only to the "peroxide" level. In other words,  $\text{CO}_2$  must be not only an acceptor for hydrogen but an essential part of an intermediate substance. (2) Photoreduction and dark reduction appear to have a great similarity as shown by the effect of poisons.

Elsewhere we have said that the photochemical processes in a green plant can be described as forcing the constituents of a molecule of water, H and OH, into a complex  $\text{Q R}(\text{CO}_2)$  whereby oxidized and reduced substances,  $\text{QOH}$  and  $\text{R(H)}(\text{CO}_2)$  are formed. On paper it is possible to do the same not photochemically but by oxidation and reduction, then we obtain



and



HDo means any hydrogen donor, in our case free hydrogen. Once the same products are formed as in photoreduction, there is no difficulty in assuming that they react further along identical lines. In this way the induced dark reduction of carbon dioxide can be described as a partial reversal of photoreduction that leads us automatically to the required maximum yield of half a molecule of carbon dioxide reduced per molecule of oxygen absorbed. A detailed discussion of the principle mentioned is only possible in close connection with a discussion of the facts observed in photoreduction and photosynthesis and therefore reserved for another communication.

#### SUMMARY

1 Unicellular algae possessing a hydrogenase system (*Scenedesmus* and other species), and having been adapted by anaerobic incubation to the hydrogen metabolism, reduce oxygen to water according to the equation  $\text{O}_2 + 2\text{H}_2 \rightarrow 2\text{H}_2\text{O}$

2 The oxyhydrogen reaction proceeds undisturbed only in the presence of carbon dioxide, which simultaneously is reduced according to the equation  $\text{CO}_2 + 2\text{H}_2 \rightarrow \text{H}_2\text{O} + (\text{CH}_2\text{O}) = (\text{carbohydrate})$

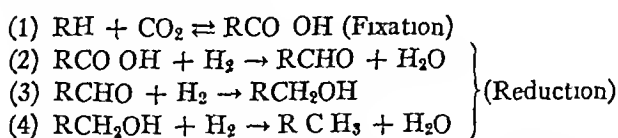
3 The maximum yield of the induced reduction is one half molecule of carbon dioxide reduced for each molecule of oxygen absorbed

4 Partial reactions are recognizable in the course of the formation of water

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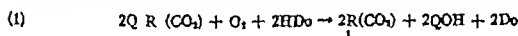
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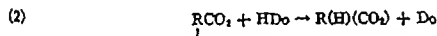
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and



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3 The maximum yield of the induced reduction is one-half molecule of carbon dioxide reduced for each molecule of oxygen absorbed.

4 Partial reactions are recognizable in the course of the formation of water

and it is with the absorption of the second equivalent of hydrogen that the carbon dioxide reduction appears to be coupled

5 The velocity of the reaction increases in proportion to the partial pressure of oxygen, but only up to a certain point where any excess of oxygen causes the inactivation of the hydrogenase system. The reaction then ends prematurely

6 During the oxyhydrogen reaction little or no oxygen is consumed for normal respiratory processes

7 Small concentrations of cyanide, affecting neither photosynthesis nor photoreduction in the same cells, first inhibit the induced reduction of carbon dioxide and then lead to a complete inactivation of the hydrogenase system

8 Hydroxylamine, added after adaptation, has either no inhibitory effect at all, or prevents solely the induced reduction of carbon dioxide without inactivating the hydrogenase system

9 Dinitrophenol prevents the dark reduction of carbon dioxide while the reduction of oxygen continues to the formation of water

10 Glucose diminishes the absorption of hydrogen, probably in its capacity as a competing hydrogen donor

11 The induced reduction of carbon dioxide can be described as an oxidation-reduction similar to that produced photochemically in the same cells

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# TYROSINASE AND PLANT RESPIRATION\*

By DWIGHT BAKER AND J. M. NELSON

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(Received for publication July 9, 1942)

It appears reasonable to assume that plant oxidases, such as tyrosinase<sup>1</sup> take part in the respiratory process in plants. This view, however, appears not to be accepted by all workers in this field. Thus Szent-Györgyi and Victorisz (1) have suggested that possibly the oxidase occurs dormant in the plant tissue and only becomes active when the tissue is injured. At such a time, it brings about the oxidation of phenolic bodies, present, in the plant, to quinones, and the latter in turn not only act in a bactericidal capacity, but also combine with protein, forming an insoluble coating over the injured tissue.

Recently Boswell and Whiting (2) have attempted to show that the oxidase in potato tubers really does play the rôle of a respiratory enzyme. They studied by means of the Warburg respirometer (3) the rates of oxygen uptake and evolution of carbon dioxide when thin potato slices were permitted to respire in the presence of water buffered with phosphate (pH about 5.5). In this way they found that the rates of oxygen uptake and evolution of carbon dioxide remained practically constant for several hours, and the respiratory quotient was close to unity. On the addition of catechol, there was a sudden marked rise in the rate of oxygen uptake. This increased rate, however, was only of short duration and was followed by a gradual drop culminating finally in a value of about one-third of the rate shown by the slices respiring in presence of phosphate only.

Adding more catechol to the reaction mixture, after this final low respiration rate had been reached, gave no further new increase in the rate of oxygen uptake, showing that all the oxidase had become exhausted or inactivated. They, therefore, attributed this lowering of the respiration rate to one-third of the normal rate in phosphate alone, to the inactivation of the oxidase, and concluded that two-thirds of the respiration of the potato slices was dependent on the oxidase present in the slices.

\* This study was aided by a grant from the Upjohn Company.

<sup>1</sup> There is a difference of opinion as to the name for this oxidase usually prepared from potato tubers and from certain varieties of mushrooms. Many workers use the terms polyphenol oxidase and catechol oxidase. The present authors prefer to adhere to the original name, tyrosinase proposed by Bertrand (*Sur une nouvelle oxydase on ferment soluble oxydant, d origine végétale Compt rend Acad sc 1896, 122, 1215*) due to the fact that this enzyme differs from other oxidases in that besides catalyzing the aerobic oxidation of polyhydric phenols it also catalyzes the oxidation of certain monohydric phenols. The terms polyphenol oxidase and catechol oxidase fail to emphasize this characteristic activity of this oxidase towards monohydric phenols.

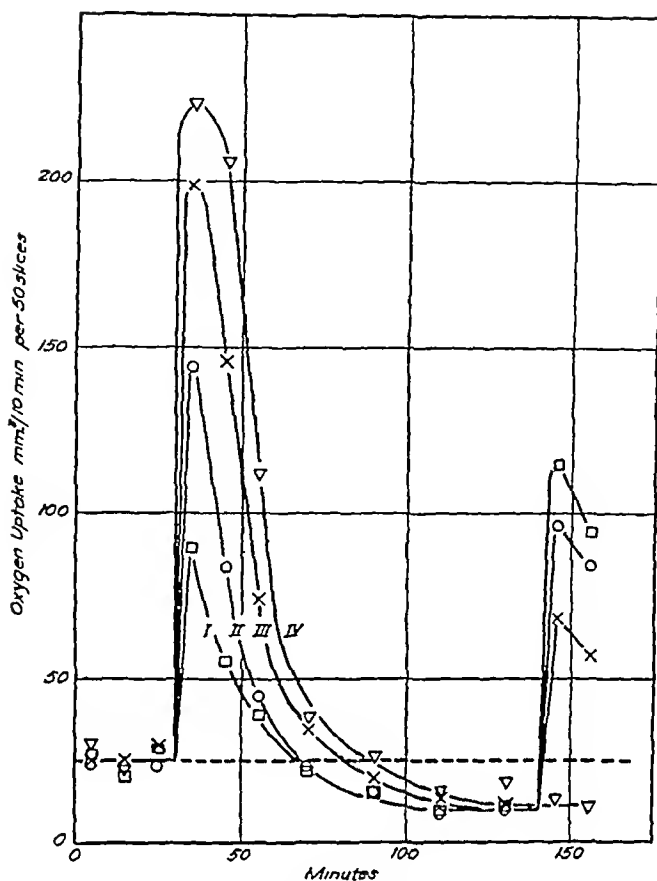


FIG 1 Showing the eventual lowering of the rate of respiration of washed potato slices in the presence of added catechol. Reaction mixtures contained in the Warburg flasks consisted of 50 slices and 5 cc of 0.04 M phosphate buffer solution. Center well contained filter paper moistened with 0.2 cc of a 20 per cent KOH solution. After the elapse of 30 minutes 1 cc of an aqueous solution containing various amounts of catechol, as specified below, was added from the side arm. Total volumes of the final reaction mixtures were 10.2 cc, pH 5.7, temperature 25°C.

All curves, I, II, III, and IV, up to the time the experiments had been in progress for 30 minutes, represent the rates of oxygen uptake by 50 slices respiring in the presence of phosphate buffer only. During the time between 30 and 140 minutes curves I, II, III, and IV show respectively the influences on the rate of oxygen uptake of 0.63, 1.25, 2.5, and 5 mg of added catechol. In each instance the final rate of oxygen uptake, due to the influence of the catechol, fell below the rate shown by the slices respiring in phosphate alone, and to the same extent irrespective of the amount of catechol added.

To decide whether or not the tyrosinase had been inactivated, during the 140 minutes in which the experiments had been in progress, the Warburg flasks were removed from the thermostat and 1 cc of a solution containing 2.5 mg of catechol was added from the side arm of each flask. The flasks were then returned to the thermostat,

Repeating the experiments of Boswell and Whiting, using conditions similar to theirs, it was found that this drop in rate of oxygen uptake to a value lower than that shown by the slices in phosphate alone cannot be attributed entirely to the removal of the oxidase activity by inactivation (see Fig 1). For example, employing an amount of catechol, insufficient to inactivate all of the oxidase in the slices, as shown by subsequent addition of more catechol, the rate of oxygen uptake still continued to drop, after the initial rise, to a value considerably below the rate shown by the slices respiring in the phosphate alone. In other words, the rate of oxygen uptake fell to practically the same low value as that observed by Boswell and Whiting, even though considerable active oxidase still remained in the slices.

That the inactivation of the oxidase cannot account for this drop in the rate of oxygen uptake below the rate when the slices respire in the presence of phosphate only, can be shown even in a more striking manner by using 4-tertiary butyl catechol. This substance, judging from observations (unpublished) made by Roth and Dawson, in these laboratories in their study of tyrosinase from the common mushroom, hardly inactivates the enzyme at all. Yet as can be seen from the data shown in Fig 2, even though only slight inactivation of the oxidase occurred, still the drop in the rate of oxygen uptake was greater than in the case of the catechol experiment (see Fig 1).

This drop in the rate of oxygen uptake to a value lower than that when the slices respired in phosphate alone therefore cannot be accounted for by the inactivation of the oxidase, but must be due to some other cause. Hence, the claim made by Boswell and Whiting that two-thirds of the respiration of the slices is dependent on the oxidase loses its main support, and leaves the question still unanswered as to whether or not the oxidase takes part in plant respiration. The present authors, however, feel that it is possible, by the procedure described below to show that tyrosinase does play the rôle of a respiratory enzyme in potato tubers.

The activity of potato tyrosinase towards protocatechuic acid (4-carboxy

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and after thermol equilibrium had been attained the rates of oxygen uptake again noted. Only in the case of the experiment corresponding to curve IV did the rate of oxygen uptake fail to respond to the addition of the second quantity of catechol. In this instance (IV) the tyrosinase due to the large amount of catechol added had apparently been completely inactivated. The renewed increase in the rates of oxygen uptake, shown by the curves I, II, and III on the addition of the second quantity of catechol shows that considerable tyrosinase activity still remained in the slices.

Taken together the four curves show that the lowering of the rate of oxygen uptake on the addition of catechol to a value below that in the presence of phosphate alone cannot be attributed to inactivation of the tyrosinase but must be due to the oxidation of catechol exerting an injurious influence on some other part of the respiratory system of the slices.

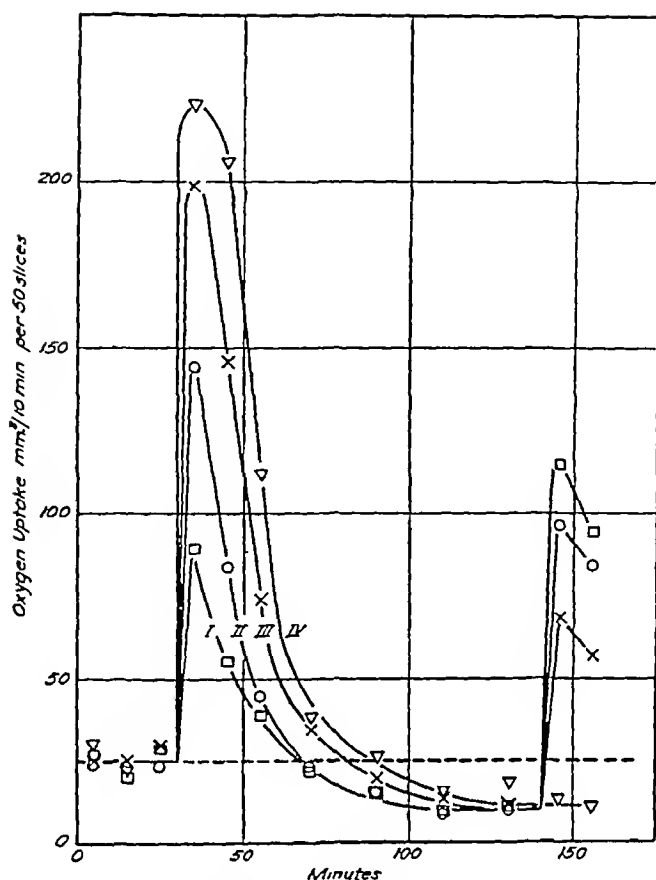


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The activity of potato tyrosinase towards protocatechuic acid

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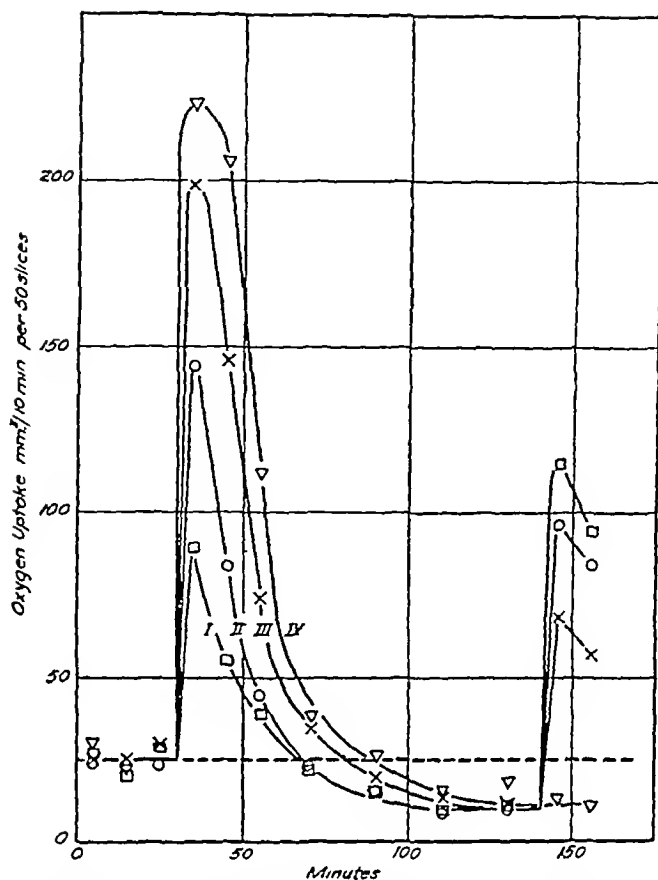


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the volume which would have been consumed by the slices if they had respired in phosphate alone, was 813  $\mu$ l. The fact that this large volume of oxygen was used up and the corresponding volume of carbon dioxide given off taken together with the fact that hardly any quinone accumulated, shows that the protocatecholic acid must have acted as a shuttle or hydrogen carrier. First it was oxidized to quinone by means of the tyrosinase, and then the quinone in turn was reduced by a hydrogen donor, adjacent to it, in the respiratory chain. Knowing that potato tyrosinase catalyzes the aerobic oxidation of protocate-

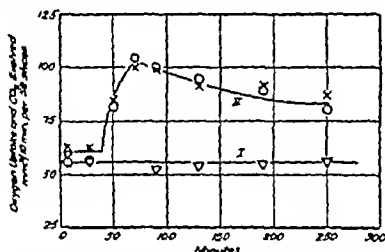


FIG. 3 Showing the influence of protocatecholic acid on the rate of respiration of potato slices. Reaction mixtures and conditions the same as those described in the legend for Fig. 1 except protocatecholic acid was used in the place of catechol. Curve I (control) shows the rate of oxygen uptake for 50 slices respiring in phosphate alone. Curve II 40 minutes after the slices had respired in the phosphate alone 2 mg. of protocatecholic acid were added from the side arm. Circles represent oxygen uptake and the crosses the  $\text{CO}_2$  values. Since the oxygen values approach closely the carbon dioxide values the  $R/Q$  is close to unity. Curve II shows that not only was the rate of oxygen uptake increased by the protocatecholic acid, but that this increased rate continues for more than 250 minutes. This is very different from the influence of catechol on the rate of oxygen uptake shown in Fig. 1.

cholic acid, coupled with the fact that the acid can take part in the respiration of the slices, it follows that potato tyrosinase can take part in the respiratory system of the slices.

That tyrosinase remains in the potato slices, after they have been subjected to being washed in running water for over 2 days, can be shown by the oxidase in the slices still retaining the characteristic ability to catalyze the oxidation of monohydroxy phenols. In Fig. 4 is shown the increase in oxygen uptake when *p*-cresol was added to the respiring slices. The retarding influence on the respiration of the slices by substances known to act as inhibitors towards tyrosinase action also indicates that tyrosinase is the active oxidase in the slices. Potassium cyanide, which is known to inhibit or retard the action of



catechol) is very much less than it is towards catechol or tertiary butyl catechol. In Fig 3 is shown the influence of this acid on the rates of oxygen uptake and carbon dioxide given off by the respiring slices. In contrast to the influences of catechol and tertiary butyl catechol (Figs 1 and 2) it will be noticed that there is a marked rise not only in the rate of oxygen uptake but also a corresponding rise in the rate of carbon dioxide given off, so that the  $RQ$  still remains, as in the case of the slices in phosphate alone, close to unity. In other words, when protocatechuic acid was added to the slices, there was not just a

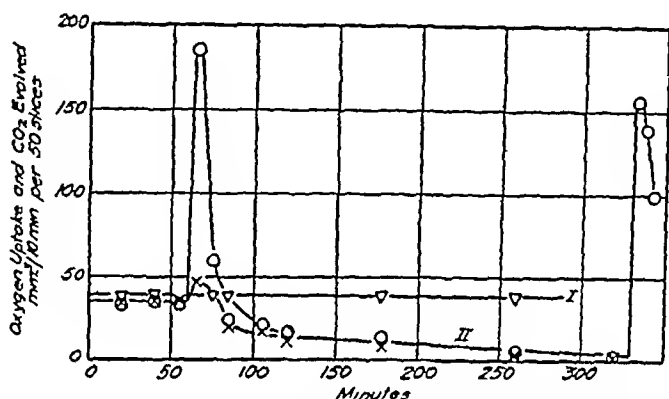


FIG 2 Showing the influence of 4-tertiary butyl catechol on the eventual rate of respiration of potato slices. Reaction mixtures and conditions same as those described in the legend for Fig 1, except 4-tertiary butyl catechol was used in place of catechol. Curve I (control) represents the rate of oxygen uptake of the slices in presence of phosphate buffer only. Curve II 60 minutes after the slices had respired in phosphate buffer only, 2.5 mg of 4-tertiary butyl catechol were added from the side arm. Circles represent oxygen uptake and the crosses the  $CO_2$  values. Just as in the case of catechol (Fig 1) a drop in the rate of oxygen uptake occurred, which extends below (approaching zero value) that in the presence of phosphate alone, even though the tyrosinase was not completely inactivated. That the tyrosinase was still active at the expiration of 330 minutes, is shown by the rise in oxygen uptake when more catechol was added.

large rise in the rate of oxygen uptake, as was the case when catechol was used, but a large rise in the rate of respiration, and instead of the rate dropping below the normal rate of the slices in phosphate alone, it remained large and comparatively steady during the 6 hours in which the experiment was in progress. Furthermore, in contrast to the catechol and tertiary butyl catechol experiments, when protocatechuic acid was used the reaction mixture only became slightly colored showing thereby very little accumulation of quinone. The calculated volume of oxygen required to convert the 2 mg of protocatechuic acid, used in the experiment, to quinone is 195  $\mu l$ . The volume taken up during the 240 minutes after the addition of the acid to the slices, deducting

the volume which would have been consumed by the slices if they had respired in phosphate alone, was 813  $\mu$ l. The fact that this large volume of oxygen was used up, and the corresponding volume of carbon dioxide given off taken together with the fact that hardly any quinone accumulated, shows that the protocatechuic acid must have acted as a shuttle or hydrogen carrier. First it was oxidized to quinone by means of the tyrosinase, and then the quinone in turn was reduced by a hydrogen donor, adjacent to it, in the respiratory chain. Knowing that potato tyrosinase catalyzes the aerobic oxidation of protocate

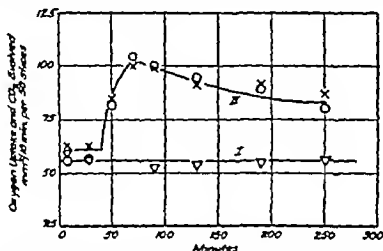


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most metal-bearing enzymes, was found to reduce the rate of respiration of the slices over 85 per cent. This, therefore, argues that a metal-bearing enzyme, such as tyrosinase, is involved in the respiratory process. 4-Nitrocatechol is known to exert a strong inhibiting action on the activity of tyrosinase (4). When this inhibitor was added to the respiring slices, it was found, as shown in Fig 5, that not only was the rate of oxygen uptake greatly decreased, but also the rate of carbon dioxide given off. In other words, retarding the tyrosinase action also retarded the respiration of the slices. The fact that both of the above retardants lowered the rate of oxygen uptake about 85 per cent

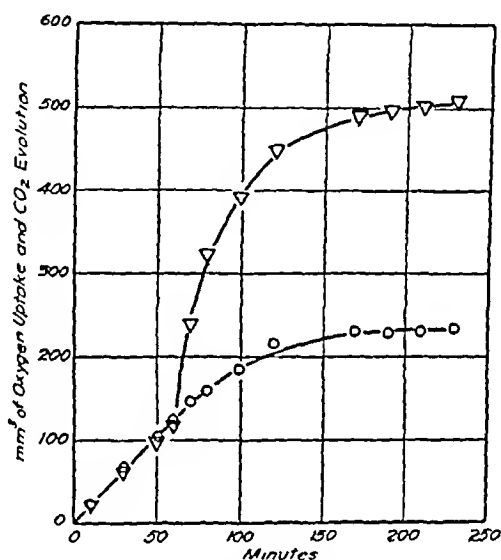


FIG 4 Showing the presence of tyrosinase in the washed potato slices. Reaction mixture and conditions the same as those described in the legend of Fig 1. Triangles represent mm<sup>3</sup> of oxygen and circles mm<sup>3</sup> of CO<sub>2</sub>. After the elapse of 60 minutes 1 mg. of *p*-cresol was added to the Warburg flasks containing the 50 slices and phosphate buffer solution.

constitutes strong evidence that the respiratory process of the slices is chiefly dependent on tyrosinase as the terminal oxidase.

The aerobic oxidation of various ortho-dihydroxy phenolic compounds not only is catalyzed at widely different rates by tyrosinase, but as has been mentioned above (Roth and Dawson), these compounds vary in their tendency to inactivate the enzyme. Catechol and tertiary butyl catechol are oxidized very fast when they are added to the respiring slices and the reaction mixtures in the Warburg flasks soon became highly colored due to the accumulation of quinones. The latter are known to be chemically very reactive, and therefore might easily exert a harmful or retarding influence on some intermediate link in the respiratory chain operating in the slices. These two catechols therefore are not suited to serve as hydrogen carriers in the respiratory system in potato

slices. On the other hand, protocatechuic acid which is oxidized more slowly, yields quinone at such a slow rate that the rest of the respiratory system is able to reduce the quinone as rapidly as it is formed, thereby enabling the acid to serve as a hydrogen carrier.

In fact, the influence of the protocatechuic acid on the respiring slices appears to be quite similar to that observed by Boswell and Whiting when they added a substance<sup>2</sup> extracted from potato tubers. They found that when this substance was added to the respiring potato slices an increase in the rate of oxygen uptake took place, and just as in the case of the addition of the protocatechuic acid, there was only a slight tendency toward a decrease in this higher rate during the 6 hours in which the experiment was conducted. The fact that this substance influenced the respiration of potato slices in much the same way as

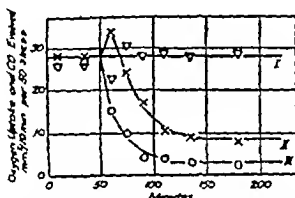


FIG. 5 Showing the inhibiting action of 4-nitrocatechol on the rate of respiration of potato slices. Reaction mixtures and conditions the same as those described in the legend for Fig. 1. Curve I (control) represents the rate of respiration of the potato slices in phosphate alone. Curve II 50 minutes after the slices had been respiring in phosphate alone 2 mg. of 4-nitrocatechol were added. Circles—oxygen uptake and crosses CO<sub>2</sub> given off.

the protocatechuic acid shows that it too functions as a hydrogen carrier. And since it was isolated from potato tubers, it probably is the natural substrate for potato tyrosinase, or at least closely related to it.

The claim made by Boswell and Whiting that one-third of the respiration of the slices is due to some other respiratory system than the one dependent on the oxidase in the slices loses weight in the light of the objection pointed out against their proof that two-thirds is dependent on the oxidase. Lowering the rate of respiration of the slices over 85 per cent by the above mentioned retardants, potassium cyanide and 4-nitrocatechol, is more in line with the view that probably all of the respiration is dependent on the oxidase, tyrosinase.

<sup>2</sup> Boswell and Whiting extracted a substance from potato tubers which possessed many of the properties common to *o*-dihydroxy phenolic compounds. It was soluble in water in alcohol, precipitated by lead acetate and gave a green color reaction with ferric chloride. They however did not identify the substance any further.

The study described above, together with that of Boswell and Whiting, on the respiration of potato tubers establishes experimentally in living tissue another terminal oxidase besides the cytochrome C oxidase

#### EXPERIMENTAL DETAILS

The potatoes used were of no particular variety. They were bought in the open market and were in good firm condition showing no tendency towards sprouting at the time. Since most of this work was done between December and May, 2 or more months had elapsed since the potatoes had been harvested.

The tubers were cut into slices of about  $1\text{ cm}^2$  and  $400\text{ }\mu$  thick. The slices were placed in running tap water and left in this running water from 20 to 100 hours. The length of time in washing the slices did not appear to change the rate of respiration. Slices from different potatoes, however, did show differences in rate of respiration. The temperature of the tap water varied from  $10$  to  $15^\circ\text{C}$ . In comparable experiments the slices were always prepared from the same potato. The dry weight of 50 slices was about  $250\text{ mg}$ .

A Warburg respirometer (3) was used for following the amount of oxygen uptake and the carbon dioxide given off. The temperature at which the respiration experiments were run was  $25^\circ\text{C}$ . The reaction flasks of the respirometer were of  $50\text{ cc}$  capacity. Fifty wet slices, weighing about  $4.5\text{ gm}$  together with  $5\text{ cc } 0.04\text{ M}$  phosphate buffer were placed in the reaction flask. To this mixture was added, usually from the side arm, water or solutions of other substances such as catechol (see legends for the figures in the text) making the final volume in the flasks  $10.2\text{ cc}$ , and the  $\text{pH} = 5.7$ . The carbon dioxide was determined by the direct method of Warburg (3), using filter paper moistened with  $0.2\text{ cc}$  of  $20\text{ per cent}$  aqueous KOH for absorbing the carbon dioxide as it was formed. The rate of shaking the Warburg apparatus was  $120$  complete oscillations per minute. The rate of respiration was independent of the rate of shaking.

#### SUMMARY

The evidence presented in this paper supports the conclusion that at least  $85\text{ per cent}$  of the oxygen uptake of the respiring tissue of potato tuber enters the chemistry of the cell by way of a tyrosinase-catalyzed oxidation.

The qualitative aspects of this conclusion are in agreement with the claim made by Boswell and Whiting. However, the evidence offered by them in support of this conclusion is shown to be inadequate.

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# ACTION OF INHIBITORS ON HYDROGENASE IN AZOTOBACTER\*

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(From the Department of Agricultural Bacteriology, University of Wisconsin Madison)

(Received for publication, August 10, 1942)

## INTRODUCTION

Previous studies on the occurrence and properties of hydrogenase in *Azotobacter vinelandii* have included a few tests with typical respiratory enzyme inhibitors, but a detailed survey of the effect of inhibitors on this enzyme has not been made. Such investigations are desirable for the following specific purposes—

1 To provide basic information concerning the nature of the enzyme system. This is essential not only for its intrinsic value but in order to compare the hydrogenase in *Azotobacter* with that in other species.

2 To compare the properties of the enzyme as it functions in the intact cell and in cell free extracts. Because  $H_2$  is such a specific substrate its oxidation by a suspension of *Azotobacter* can be readily measured with a minimum of complications arising from concurrent oxidations of other substrates. This *Knallgas* reaction is also catalyzed by cell-free enzyme preparations (Lee, Wilson, and Wilson, 1942). An opportunity is thus furnished to test with an oxidative enzyme system the constantly disputed question as to whether results obtained with cell free enzymes are applicable to biochemical reactions in the intact cell.

3 To discover, if possible, an inhibitor with differential effects on the oxidation of hydrogen and of other substrates. Although concomitant respiratory activity is not a serious difficulty in studies with *azotobacter*, it becomes so with suspensions of the root nodule bacteria (*Rhizobium*, sp.) taken directly from the nodules of leguminous plants. Such suspensions retain a high "endogenous" respiration in spite of repeated washings (Thorne and Burris, 1940). For this reason demonstration of a hydrogenase is difficult. Because of its significance for the mechanism of fixation (Lee, Wilson, and Wilson, 1942), more extensive information respecting the occurrence of hydrogenase in nodule suspensions should be secured.

## Methods

Details of the method for measuring hydrogenase activity in cultures of *Azotobacter vinelandii* have been described by Wilson, Lee, and Wilson (1942).

\* This research was supported in part by grants from The Rockefeller Foundation and from the Wisconsin Alumni Research Foundation.

Essentially, the gas uptake in a  $H_2$ - $O_2$  mixture by a "resting" suspension which contains about 0.1 mg N is measured at  $35^\circ C$  in a Warburg microrespirometer. The rate, which remains constant over the period of the run (about 60 minutes), is calculated as the  $Q_N(N) = c \text{ mm}$  total gas uptake per hour per mg cell N. A suitable control on respiration is made in air or in a  $He$ - $O_2$  mixture, and the rate calculated as the  $Q_{O_2}(N) = c \text{ mm}$   $O_2$  uptake per hour per mg cell N.

## EXPERIMENTAL

*Action of Inhibitors on Oxidation of  $H_2$  and Other Substrates by Azotobacter*

**Cyanide**—Lee *et al.* (1942) reported that  $m/1000$  KCN at pH 7.5 completely inhibited the hydrogenase in *Azotobacter*. This has been confirmed in two

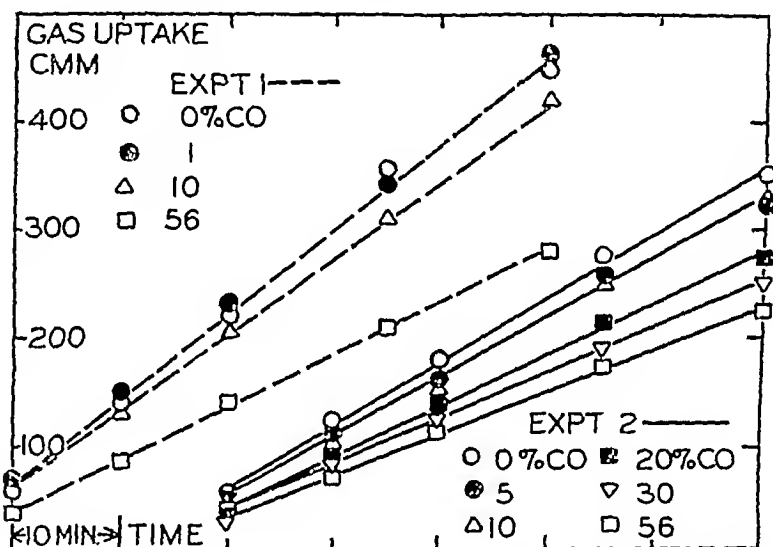


FIG. 1. Effect of CO on oxidation of hydrogen by *Azotobacter vinelandii*. Gas mixture  $p_{H_2}$ , 0.4;  $p_{O_2}$ , 0.04 atm, indicated CO and helium to 1 atm.

additional experiments indicating that the Knallgas reaction is entirely cyanide-sensitive.

**Carbon Monoxide**—The oxidation of hydrogen by *Azotobacter* is likewise very sensitive to CO (Fig. 1). The composition of the gas mixtures in these experiments was 4 per cent  $O_2$ , 40 per cent  $H_2$ , the indicated quantity of CO, and He to one atmosphere. A definite effect on the rate of hydrogen oxidation is observed with as little as 5 to 10 per cent CO, and with 56 per cent CO the rate may be inhibited as much as 50 per cent. In Experiment 1 the  $Q_N(N)$  values for different ratios of CO/ $O_2$  (given in parentheses) were (0), 4900, (1/4), 4900, (5/2), 4500, (14/1), 3300. In Experiment 2 these were (0), 5140, (5/4), 4750, (5/2), 4720, (5/1), 4000, (15/2), 3430, (14/1), 3140. Controls in which helium was substituted for  $H_2$  demonstrated that respiration on

glucose or succinate was inhibited slightly, if at all, even with 56 per cent CO in the gas mixture.

Attempts to reverse the CO inhibition with light were unsuccessful. Recently, Dr H. D. Hoberman informed us in a private communication that he had observed a similar CO inhibition which was light reversible with a hydrogenase in *Proteus vulgaris*. Other information from Dr Hoberman indicated that the enzyme in *Proteus* is similar in many of its properties to that

TABLE I

*Inhibition of Respiratory and Hydrogenase Activity in Azotobacter vinelandii by Sodium Azide*

Experiment No	Concentration of azide	Respiration		Hydrogenase	
		Q <sub>o2</sub> (N)	Inhibition	Q <sub>H</sub> (N)	Inhibition
1	None	—	—	3200	—
	M/100	—	—	2200	31
2	None	—	—	3330	—
	M/100	—	—	2530	24
3	None	3560	—	4320	—
	M/4500	3280	8	4320	0
	M/900	2630	26	4540	-5
	M/450	1190	66	4307	0
	M/90	1380	71	3380	22
	M/9	57	98	1077	75
4	None	4200	—	2100	—
	M/10 000	4200	0	—	—
	M/5000	4250	-1	3160	-50
	M/1000	3320	21	3020	-44
	M/500	2440	42	2660	-27
	M/100	430	89	1410	33
	M/10	113	97	300	86

Respiration M/150 sucrose in air

Hydrogenase no added substrate in 96 per cent H<sub>2</sub>, 4 per cent O<sub>2</sub>

in *Azotobacter*. It appears likely, then, that the CO inhibition of hydrogenase in *Azotobacter* is also light-sensitive. Additional experiments are in progress to investigate this point in detail.

*Sodium Azide.*—Lee *et al* (1942) found that sodium azide at its optimum pH (6.5) was less inhibitory than cyanide. This has been verified in further trials as illustrated by the data in Table I. With low concentrations of azide stimulation is frequently observed, at M/100 there is an inhibition of about 25 per cent which increases to 75 to 85 per cent at M/10. A marked differential inhibition of the oxidation of H<sub>2</sub> and other substrates is obtained in the range M/1000 to M/100.



*Hydroxylamine*—Data in Fig 2 and Table II demonstrate that hydroxylamine has a pronounced differential inhibitory effect on the respiration and hydrogenase activity of *A. vinelandii*. Whereas respiration on glucose and

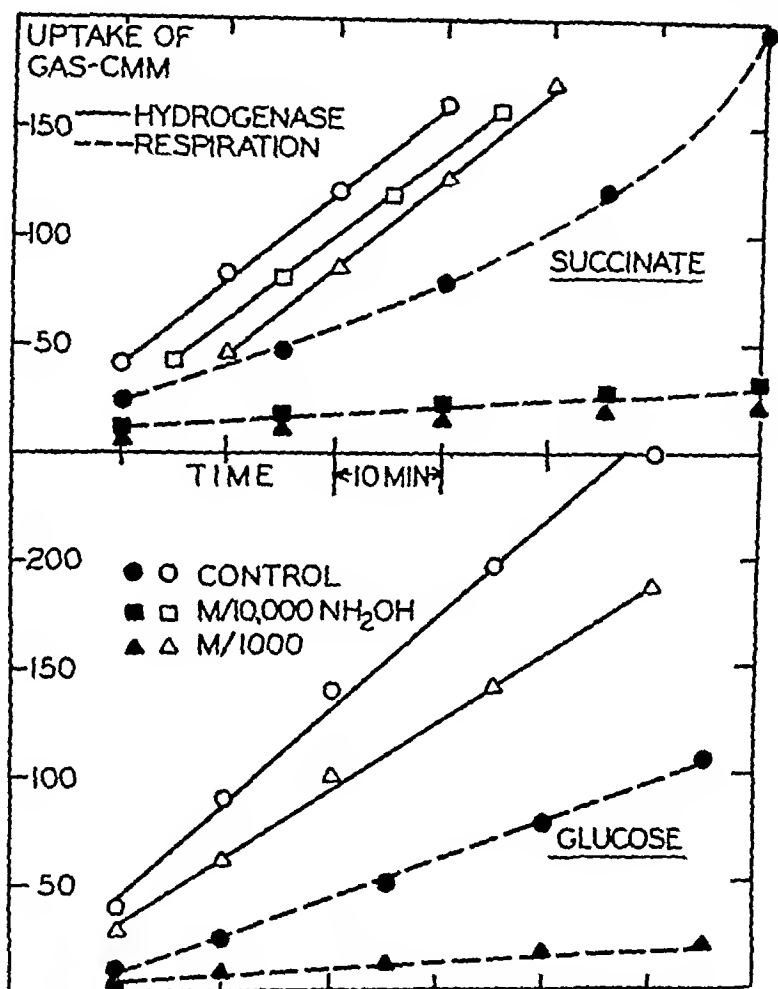


FIG 2 Inhibition by hydroxylamine of respiration and hydrogenase in *Azotobacter vinelandii*. Hydrogenase measured in 96 per cent  $H_2$ , 4 per cent  $O_2$ , respiration, in air. Upper, succinate as respiration substrate, no effect of  $NH_2OH$  on hydrogenase. Lower, glucose as substrate,  $Q_K(N)$  in absence of  $NH_2OH$ , 2260, in presence, 1740. Legend applies to both sections of figure.

succinate may be almost entirely suppressed with  $M/1000$  to  $M/500$   $NH_2OH$ , oxidation of  $H_2$  is not markedly reduced (0 to 25 per cent). If pyruvate is supplied the organism, the inhibition of respiration disappears, undoubtedly because the  $NH_2OH$  is detoxified by combination with the carbonyl group of the substrate.

*Iodoacetate and Fluoride*—The results with sodium fluoride and iodoacetate are given in Table III. Differential inhibition of respiration and hydrogen oxidation by both is definite, but is especially outstanding with  $\mu/100$  sodium

TABLE II

*Effect of  $\text{NH}_4\text{OH}$  on Respiration and Hydrogenase in *Azotobacter vinelandii**

Experiment No	Concentration of inhibitor	Respiration		Hydrogenase	
		$Q_{O_2}(N)$	Inhibition	$Q_H(N)$	Inhibition
5	None	4000	—	3520	—
	$\mu/10\ 000$	370	90	3680	-4
	$\mu/5000$	350	90	3460	0
	$\mu/1000$	350	90	3580	0
	$\mu/500$	75	98	3380	4
	$\mu/100$	180	95	500	86
6	None	1560	—	2590	—
		2150			
	$\mu/1000$	0	100	1940	25

Respiration  $\mu/100$  succinate in air in Experiment 5  $\mu/150$  succinate in 96 per cent  $\text{H}_2$ , 4 per cent  $\text{O}_2$  in Experiment 6.

Hydrogenase no added substrate, 96 per cent  $\text{H}_2$ , 4 per cent  $\text{O}_2$  in both experiments.

TABLE III

*Action of Iodoacetate and Fluoride on Respiration and Hydrogenase in *Azotobacter vinelandii**

Inhibitor	Concentration	Respiration		Hydrogenase	
		$Q_{O_2}(N)$	Inhibition	$Q_H(N)$	Inhibition
None	—	9560	—	4340	—
Iodoacetate	$\mu/1000$	5860	39	4500	0
	$\mu/100$	760	92	4300	0
Fluoride	$\mu/25$	9900	-4	4710	-8
	$\mu/12\ 5$	6400	34	4410	0

Respiration  $\mu/150$  glucose in air

Hydrogenase no added substrate in 96 per cent  $\text{H}_2$ , 4 per cent  $\text{O}_2$

*iodoacetate* The absence of inhibition with both fluoride and iodoacetate suggests that the oxidation of  $\text{H}_2$  does not involve a phosphorylation step

#### *Hydroxylamine As a Specific Inhibitor for Biological Nitrogen Fixation*

The experiments with  $\text{NH}_4\text{OH}$  explain some curious results reported a few years ago by Kubo (1937) who found that, as the concentration of this inhibitor was varied from  $\mu/10,000$  to  $\mu/250$  oxygen uptake by suspensions of

*Azotobacter chroococcum* on mannitol was materially decreased in air but not in a mixture of 80 per cent  $H_2$  and 20 per cent  $O_2$ . He interpreted this finding as evidence for a *specific* inhibition of nitrogen fixation by  $NH_2OH$  which might be regarded as indirect support for the view that hydroxylamine is the key intermediate in the chemical mechanism. Burk was unable to confirm this specific inhibition and has questioned the validity of the hydroxylamine hypothesis for nitrogen fixation by *Azotobacter* (Burk, 1937, Burk and Burns, 1941, Burk and Horner, 1935).

Kubo's results now admit a more logical but less spectacular explanation, *viz.*, that he unknowingly was dealing with the differential effect of  $NH_2OH$  on respiration and hydrogen oxidation by *Azotobacter*. Unfortunately, he did not include an exact control (flask to which no inhibitor was added), but it appears probable that his experiments are readily and simply explained as follows. In the air series at the lowest concentration of  $NH_2OH$  (M/10,000) there remained considerable residual respiration which was effectively eliminated on further additions of the inhibitor. In the hydrogen-oxygen series, however, the gas uptake in the presence of M/10,000  $NH_2OH$  represents the total for two competing reactions: oxidation of mannitol and of  $H_2$ . As the respiration in this series is inhibited by additional  $NH_2OH$ , the decrease in  $O_2$  consumed is replaced by a more or less equivalent quantity of  $H_2$  and  $O_2$  which disappears in the *Knallgas* reaction.

If the foregoing explanation is correct, the same type of data should be obtained with any differential inhibitor. As is illustrated in Fig. 3, this is actually the case. In these experiments we have used the proper control with the following results: respiration of mannitol is almost completely inhibited by M/1000  $NH_2OH$  or M/500 sodium azide, a compound which hitherto, at least, has not been suggested as an intermediate in biological nitrogen fixation. The reduction in gas uptake in the  $H_2$ - $O_2$  mixture is much smaller than in air since only the part due to oxidation of mannitol has been eliminated, the oxidation of  $H_2$  probably remaining essentially the same. This leads to a spurious "specific" inhibition of biological nitrogen fixation, hence this particular support for the hydroxylamine hypothesis will have to be discarded.

#### *Detection of Hydrogenase in Organisms with High Endogenous Respiration*

The differential inhibitors allow a modification of our previously described method for detection of hydrogenase (Wilson, Lee, and Wilson, 1942) which should be useful with organisms in which the endogenous respiration is high. Preliminary trial of the modified method was made with *Azotobacter* cells in order to test a species known to contain the enzyme. A gas mixture of 80 per cent  $H_2$  and 20 per cent  $O_2$  was compared with air, and no special precautions were taken to remove substrate by repeated washings.

In agreement with our previous observations hydrogenase activity is appre-

ciably decreased in the presence of 20 per cent  $O_2$  unless a high concentration of cells is used (Table IV) Although the cells were not repeatedly washed,

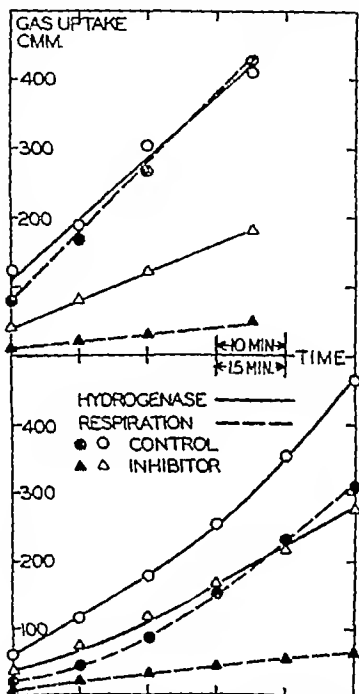


FIG. 3. Action of hydroxylamine and sodium azide on oxidation of mannitol and hydrogen by *Azotobacter vinelandii*: Respiration on  $\mu/100$  mannitol in air, hydrogenase on  $\mu/100$  mannitol in 80 per cent  $H_2$ —20 per cent  $O_2$ . Upper  $\mu/1000$   $NH_2OH$  as inhibitor. Lower  $\mu/500$   $NaN_3$  as inhibitor. Legend applies to both sections.

the respiration was small in comparison with the gas uptake due to oxidation of  $H_2$ . Even under these conditions the differential effect of the inhibition by  $NH_2OH$  is evident.

The usefulness of the modification is best illustrated by the data of Experi

ment 10 in which the medium used to wash the cells from the agar was employed as the suspending fluid. A respirable substrate is thus available, and as already noted (Wilson *et al*, 1942) in this case an active hydrogen oxidation

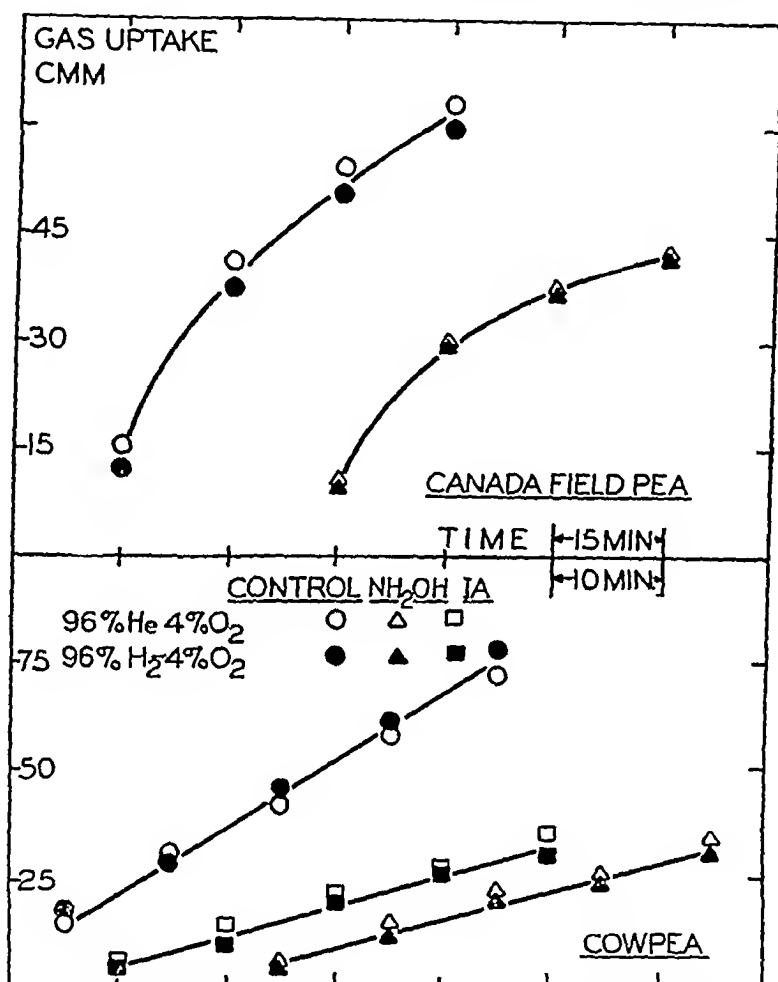


FIG. 4. Test for hydrogenase in suspensions from nodules of pea and cowpea using differential inhibitors. Hydrogenase measured in 96 per cent H<sub>2</sub>, 4 per cent O<sub>2</sub>, respiration control in 96 per cent He, 4 per cent O<sub>2</sub>, no added substrate. Pea, 0.3 mg N per flask, cowpea, 0.8 mg N per flask. Legend applies to both sections, IA—sodium iodoacetate.

occurs even with a low concentration of cells and a  $pO_2$  of 0.2 atm. The hydrogenase activity, however, is partially obscured by the accompanying high rate of respiration. If  $M/1000$  NH<sub>2</sub>OH is added, the  $Q_K$  is hardly affected, but the  $Q_{O_2}$  is reduced appreciably so that the presence of a hydrogenase is unmistakably demonstrated. Although the modified method allows the *Azoto-*

bacter suspension to be made directly from the growth on agar and the use of air for the respiration control, it would not be particularly advantageous with this organism. It should be of definite value, however, with suspensions from nodules since the bacteria in these have a gum coating which serves as substrate.

Data in Fig 4 illustrate the use of the modified method. Suspensions of root nodule bacteria from pea and cowpea nodules, prepared by the method of Thorne and Burns (1940) were tested for hydrogenase. The relatively high

TABLE IV

*Effect of  $\text{NH}_4\text{OH}$  on Respiration and Hydrogenase in Washed and Unwashed Cells of Azotobacter*

Experiment No.	Species	Treatment	Cells <i>mg N/flask</i>	$\mu/1000$ $\text{NH}_4\text{OH}$	$\text{O}_2$ (N)	$\text{O}_2$ (N)
8	<i>vinelandii</i>	Unwashed	0.32	—	160	4930
				+	134	4770
				—	113	4550
	<i>chroococcum</i>	Unwashed	0.19	—	276	4650
				+	182	5260
—				113	4460	
9	<i>vinelandii</i>	Washed	0.03	—	76	710
		Unwashed		+	18	710
				—	360	1040
				+	43	1325
				—	2000	3000
10	<i>vinelandii</i>	Unwashed + supernatant	0.09	—	2000	3000
		Washed		+	1240	2890
				—	133	666
				+	66	555
				—	—	—

Respiration in air hydrogenase in 80 per cent  $\text{H}_2$ , 20 per cent  $\text{O}_2$

Unwashed growth on agar suspended in Burk's salt solution and centrifuged supernatant discarded, and dilution made with fresh Burk's salt solution.

Washed cells washed two additional times in centrifuge tube before dilution.

endogenous respiration is largely eliminated by  $\text{NH}_4\text{OH}$  or iodoacetate. The test for hydrogenase is negative since both the total gas uptake and the effect of the inhibitors is the same in the  $\text{H}_2\text{-O}_2$  as in the  $\text{Hc-O}_2$  mixture. The results with the suspension from pea do not entirely agree with previous ones obtained with the methylene blue technique (Phelps and Wilson, 1941) since in several instances positive results have been observed with this organism. Previous tests on bacteria taken from the soybean nodule, however, have been negative even with the methylene blue technique. This problem is being further investigated using an improved methylene blue method together with the modified gasometric method.

## SUMMARY

The inhibitors usually associated with the activity of the cytochrome oxidase system—cyanide and carbon monoxide—are also effective in reducing the oxidation of  $H_2$  by intact cells of *Azotobacter vinelandii*. The hydrogenase system is more sensitive to CO than is the respiratory system.

Oxidation of a carbon source and of hydrogen by *Azotobacter* cells is inhibited in a quantitatively different manner by the following compounds: sodium azide, hydroxylamine, sodium iodoacetate, and sodium fluoride. In every case, a concentration range which is definitely inhibitory for respiration has little or no effect on the hydrogenase activity.

The differential inhibition by hydroxylamine explains certain observations in the literature which have been erroneously interpreted as demonstrating a specific inhibition by  $NH_2OH$  of biological nitrogen fixation. This supposed demonstration has been offered as support for the hypothesis that  $NH_2OH$  is an intermediate in the fixation reaction.

The differential inhibitors can be used for detection of hydrogenase in cultures possessing a high endogenous respiration. The method is illustrated by an experiment with root nodule bacteria from pea and cowpea nodules. No hydrogenase was found in either.

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# THE ELECTRICAL CONDUCTIVITY OF SODIUM AND POTASSIUM GUAIACOLATES IN GUAIACOL

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## INTRODUCTION

Measurements on the electrical conductivity at 25°C of sodium and potassium guaiacولات in guaiacol, which was nearly saturated with water, were reported in this journal some years ago<sup>1</sup>. From these measurements values of the limiting equivalent conductances and of the ionization constants were obtained for these salts. The calculations involved the simultaneous solution of three equations, namely, a cubic conductance equation, a quadratic mass law equation, and a logarithmic activity coefficient equation. The mathematical treatment used had been described by Fuoss and Kraus.<sup>2</sup> A simpler treatment was subsequently published by Fuoss,<sup>3</sup> and the author<sup>4</sup> published a simplification of the method of computation, by replacing the cubic conductance equation with a quadratic.

A recomputation of our data on the conductance of sodium and potassium guaiacولات by this improved method leads to somewhat different values for the limiting conductances and ionization constants. In this communication the new computation will be given and it will be shown that the constants so derived result in equations which hold to higher concentrations than had been formerly reported.

## THEORETICAL

The equations used in the computations of the conductance data are the following<sup>4</sup>:

A modified form of the Onsager equation for weak electrolytes,

$$\frac{\theta}{A} = \frac{1}{A_0} + \left( \frac{\alpha A_0 + \beta}{A_0^2} \right) \sqrt{C\theta} \quad (1)$$

the law of mass action,

$$K = \frac{C\theta^2\gamma^2}{(1-\theta)} \quad (2)$$

<sup>1</sup> Shedlovsky, T. and Uhlig, H. H. *J. Gen. Physiol.* 1933-34, 17, 549

<sup>2</sup> Fuoss, R. M., and Kraus, C. A. *J. Am. Chem. Soc.* 1933, 55, 476

<sup>3</sup> Fuoss, R. M. *J. Am. Chem. Soc.* 1935 57, 488.

<sup>4</sup> Shedlovsky, T. *J. Franklin Inst.* 1938 225, 739



a convenient parameter defined by

$$Z = \frac{\alpha\Lambda_0 + \beta}{\Lambda_0^{3/2}} \sqrt{C\Lambda} \quad (3)$$

and another convenient parameter,

$$F = 1 + Z + \frac{Z^2}{2} \quad (4)$$

In these equations,  $\Lambda$  is the equivalent conductance at the concentration,  $C$ ,  $\Lambda_0$  is the corresponding value at  $C = 0$ ,  $\theta$  is the degree of ionization,  $\gamma$  is the mean ionic activity coefficient,  $K$  is the ionization constant, and  $\alpha$  and  $\beta$  are theoretical constants of the Onsager conductance equation, which are 2.93 and 19.36 respectively for water-saturated guaiacol at 25°

Combining equations (1), (3), and (4) there results

$$\theta = \frac{\Lambda F}{\Lambda_0} \quad (5)$$

which, with equation (2), yields

$$\Lambda F = \Lambda_0 - \frac{C\Lambda^2 F^2 \gamma^2}{K\Lambda_0} \quad (6)$$

or

$$\frac{1}{\Lambda F} = \frac{1}{\Lambda_0} + \frac{C\Lambda F \gamma^2}{K\Lambda_0^2} \quad (6')$$

The activity coefficient,  $\gamma$ , is given by the Debye-Huckel equation

$$-\log \gamma = a\sqrt{C\theta} \quad (7)$$

in which the constant  $a$  has the value 6.52 for water-saturated guaiacol at 25°

When a suitable value of  $\Lambda_0$  is chosen, a plot of  $\frac{1}{\Lambda F}$  vs  $C\Lambda F \gamma^2$  should from equation (6') be linear, extrapolating to  $\frac{1}{\Lambda_0}$ . The corresponding value of  $K$  is obtained from the slope,  $\left(\frac{1}{K\Lambda_0^2}\right)$

#### RESULTS AND DISCUSSION

The results of the new computations are listed in Tables I, II, and III. I am greatly indebted to Dr. W. J. V. Osterhout for new measurements on the conductance of sodium guaiacolate extending to higher concentrations than we had measured. They are listed in Table III. The corresponding plots of

$\frac{1}{\Lambda F}$  vs  $C\Lambda F \gamma^2$  are shown in Fig. 1

It will be noted that the points at higher concentrations exhibit considerable curvature from the straight line. This is probably due to failure of the "limiting law" for activity coefficients, equation (7), at these values. However,

TABLE I  
*Potassium Guaiacolate,  $A_2 = 9.5$*

C (mole/liter)	$0.412 \times 10^{-3}$	$0.959 \times 10^{-3}$	$2.151 \times 10^{-3}$	$1.719 \times 10^{-3}$	$3.763 \times 10^{-3}$
A	2.461	1.808	1.300	1.037	0.862
F	1.055	1.069	1.089	1.105	1.120
$\theta$	0.2732	0.2035	0.1490	0.1206	0.1017
$1/AF$	0.385	0.517	0.706	0.873	1.036
$\gamma^2$ (equation 7)	0.719	0.657	0.584	0.529	0.483
$\gamma^2$ (equation 7')	0.734	0.680	0.616	0.569	0.530

TABLE II  
*Sodium Guaiacolate,  $A_2 = 9.0$*

C (mole/liter)	$0.20 \times 10^{-3}$	$0.771 \times 10^{-3}$	$1.24 \times 10^{-3}$	$4.34 \times 10^{-3}$	$9.81 \times 10^{-3}$	$16.46 \times 10^{-3}$
A	2.893	1.707	1.097	0.809	0.601	0.460
F	1.042	1.063	1.088	1.108	1.132	1.159
$\theta$	0.3350	0.2017	0.1325	0.0996	0.0756	0.0593
$1/AF$	0.332	0.551	0.838	1.116	1.469	1.874
$\gamma^2$ (equation 7)	0.779	0.688	0.596	0.528	0.457	0.389
$\gamma^2$ (equation 7')	0.788	0.706	0.626	0.568	0.509	0.453

TABLE III  
*Sodium Guaiacolate,  $A_2 = 9$*

C (mole/liter)	$4.49 \times 10^{-3}$	$9.18 \times 10^{-3}$	$20.19 \times 10^{-3}$	$30.46 \times 10^{-3}$	$49.99 \times 10^{-3}$	$98.3 \times 10^{-3}$
A	0.811	0.595	0.426	0.356	0.283	0.204
F	1.107	1.133	1.169	1.192	1.222	1.269
$\theta$	0.0998	0.0750	0.0553	0.0471	0.0384	0.0288
$1/AF$	1.114	1.482	2.008	2.357	2.893	3.864
$\gamma^2$ (equation 7)	0.530	0.455	0.367	0.321	0.268	0.203
$\gamma^2$ (equation 7')	0.570	0.507	0.434	0.397	0.354	0.300

if instead of equation (7), we use the Debye-Hückel "first approximation" equation,

$$-\log \gamma = \frac{\sigma \sqrt{C\theta}}{1 + b \sqrt{C\theta}} \quad (1)$$

which reduces to equation (7) at low concentrations, most of the curvature in

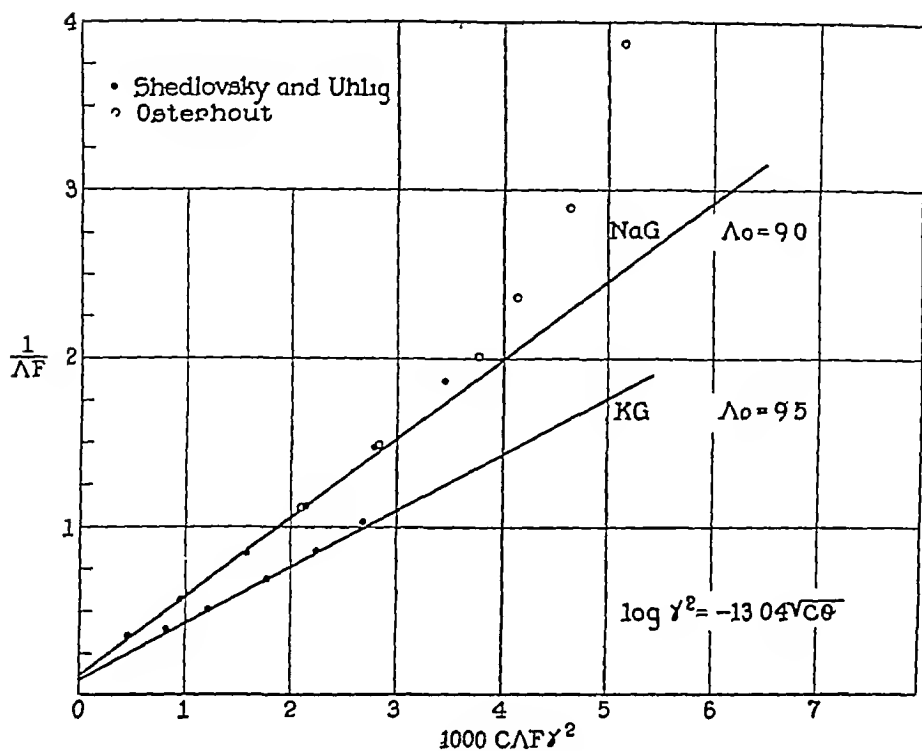


FIG 1 Equations (6') and (7)

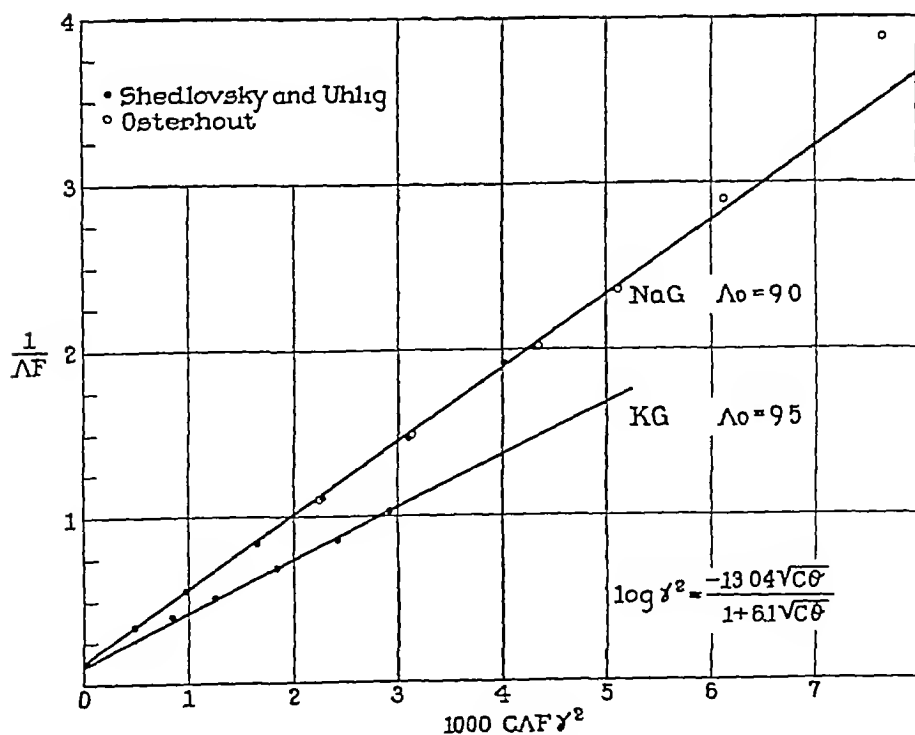


FIG 2 Equations (6') and (7')

the plot of  $\frac{1}{\Delta F} \approx C\Delta F\gamma^2$  can be removed, as is shown in Fig 2. The empirical constant  $b$  of equation (7') depends on the average ionic diameter for the salt. For these guaiacol solutions  $b \approx 0.766 \text{ \AA}$ , the factor  $A$  being expressed in

TABLE IV

	$\Delta_s$		$K$	
	Old	New	Old	New
KG	8.0	9.5	$5.1 \times 10^{-3}$	$3.4 \times 10^{-3}$
NaG	7.5	9.0	$4.3 \times 10^{-3}$	$2.8 \times 10^{-3}$

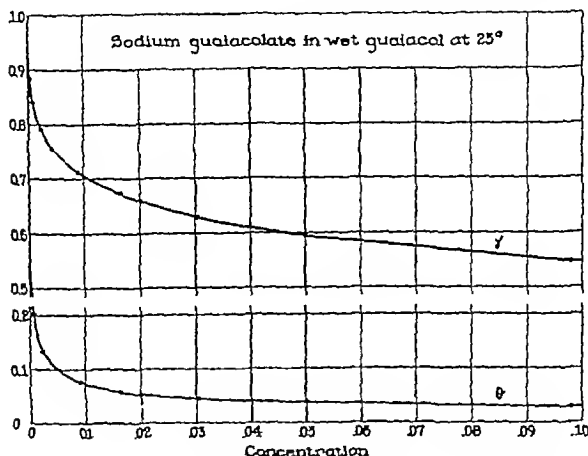


FIG 3 Dependence of activity coefficient and of degree of ionization on concentration.

Ångström units. The value of  $b = 6.1$  was used in the computations for preparing the plot in Fig 2. This corresponds to a reasonable value of the mean ionic diameter of about  $8 \text{ \AA}$ . Here it should be stated that the factor

$\frac{1}{1 + b\sqrt{C\theta}}$  in the activity coefficient equation tends to take into account ion-ion interactions which are also involved in the mass action formulation, as has

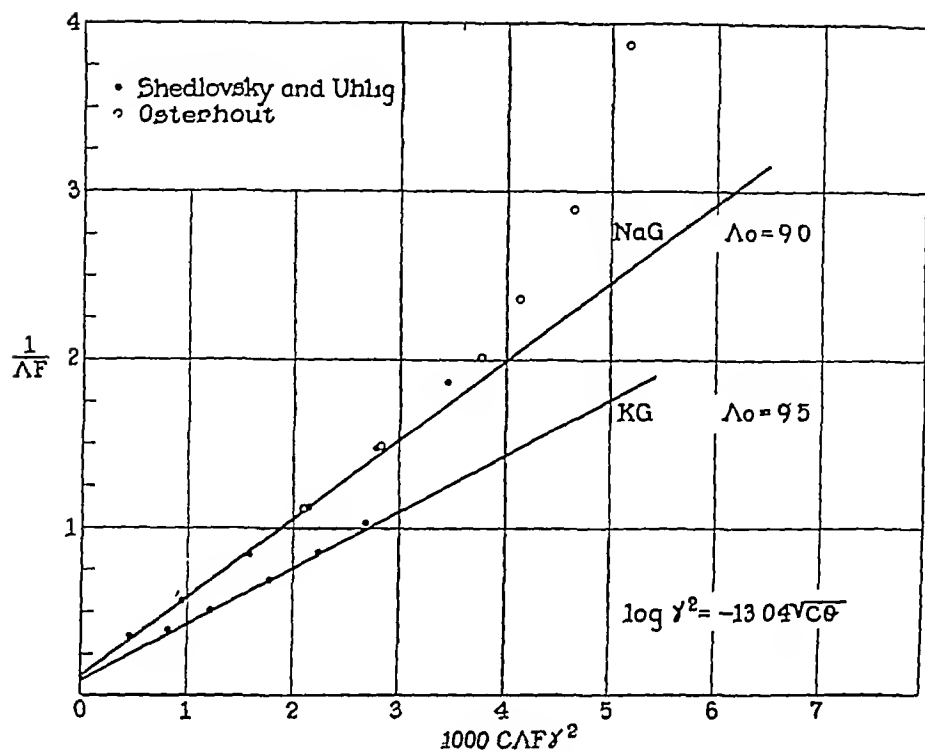


FIG 1 Equations (6') and (7)

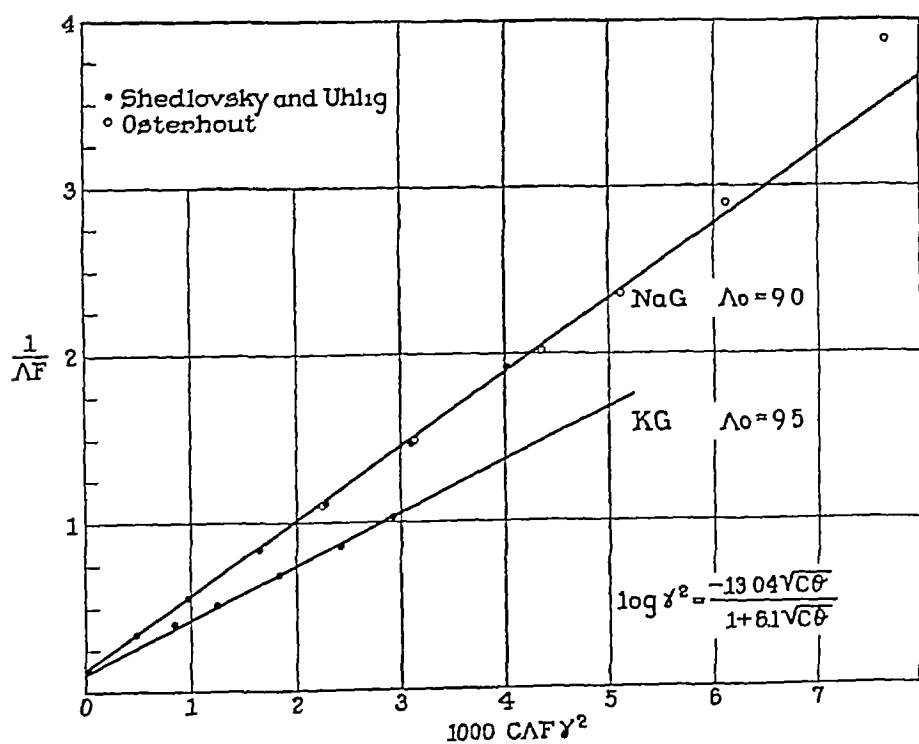


FIG 2 Equations (6') and (7')

# DIFFUSION POTENTIALS IN MODELS AND IN LIVING CELLS

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(Received for publication, October 15, 1942)

It is important to learn what fundamental properties of protoplasm are responsible for its electrical behavior. Progress in this field evidently depends on advances in physical chemistry and their use in biology.

Experiments on cells which are especially suitable for such studies show that they possess the properties of an aqueous system covered by a thin layer of non-aqueous material which is the chief seat of the electrical potentials. This material is present in exceedingly small amounts so that we can hardly hope to obtain enough for analysis. Failing this we may try to find models which act like the living cell. Much has been learned in this way.

A useful substance for this purpose is guaiacol which acts like certain protoplasmic surfaces in various ways, such as the following:<sup>1</sup>

- 1 It allows water to pass freely: it admits inorganic electrolytes and to a still greater extent certain "lipoid soluble" substances.

- 2 It is more permeable to potassium salts than to sodium salts and more permeable to chlorides than to sulfates.

- 3 When it is shaken with 0.01 M NaCl and placed in a U tube with aqueous 0.1 M NaCl on one side and aqueous 0.01 M NaCl on the other the dilute solution is electrically positive in the external circuit. This indicates that the mobility of  $\text{Na}^+$  (i.e.,  $u_{\text{Na}}$ ) is greater than that of  $\text{Cl}^-$  (i.e.,  $v_{\text{Cl}}$ ). This applies also to KCl and to the guaiaculates of sodium and potassium (which will be called for convenience KG and NaG).

- 4 When aqueous 0.1 M KCl is placed on one side of guaiacol (previously shaken with 0.1 M NaCl) and aqueous 0.1 M NaCl is placed on the other the KCl is negative in the external circuit ("potassium effect"). This indicates that  $u_{\text{K}}$  is greater than  $u_{\text{Na}}$ . This applies also to KG and NaG.

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<sup>1</sup> Cf. Osterhout, W J V. Some models of protoplasmic surfaces, in Cold Spring Harbor symposia on quantitative biology. Cold Spring Harbor Long Island Biological Association 1940 8, 51.

<sup>2</sup> Shedlovsky T. and Uhlig H. H. *J Gen Physiol*, 1933-34 17, 549-563.

been pointed out by Fuoss<sup>2</sup> It is, however, largely an empirical term, and its inclusion in the computations makes it possible to extend the validity of equation (6') to higher concentrations The values we formerly published<sup>1</sup> for the constants of sodium and potassium guaiacolate in water-saturated guaiacol at 25° and the recomputed values are given in Table IV

The discrepancy in  $K$  which appears to be large is mostly illusory, however, since the product of  $K$  and  $\Lambda_0^2$  is the significant term in the equations, and this product has been altered but little

It may appear disturbing to contemplate the new values for  $K$  in connection with partition coefficient data on these salts, distributed between water and guaiacol, which we had previously reported<sup>5</sup> However, here again, the important quantity is  $S_0^2/K$ , in which  $S_0$  is the limiting partition coefficient Here, the new value of  $S_0$  for potassium guaiacolate is  $S_0 = 0.0127$  instead of 0.0153, and for sodium guaiacolate  $S_0 = 0.00713$  instead of 0.00855 The agreement between the observed and calculated values of the salt concentrations in the non-aqueous phase (Shedlovsky and Uhlig, Tables I and II<sup>6</sup>) is, if anything, improved

It may be of interest to show how marked is the dependence of the degree of ionization,  $\theta$ , and of the activity coefficient,  $\gamma$ , on concentration,  $C$ , for these salts, which are weak electrolytes in guaiacol, although they are strong electrolytes in water Plots of  $\theta$  vs  $C$  and of  $\gamma$  vs  $C$  are shown in Fig. 3 for sodium guaiacolate

I wish to express my thanks to Dr. W. J. V. Osterhout for making his measurements on sodium guaiacolate available to me, and for stimulating discussion

#### SUMMARY

The data of the author and Uhlig, and new data, on the conductivity of sodium and of potassium guaiacolate in guaiacol at 25° have been computed with an improved conductance equation which is valid to somewhat higher concentrations than the equations formerly used

The new constants are,  $\Lambda_0 = 9.0$ ,  $K = 2.8 \times 10^{-5}$  for sodium guaiacolate and  $\Lambda_0 = 9.5$ ,  $K = 3.4 \times 10^{-5}$  for potassium guaiacolate

<sup>5</sup> Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933-34, 17, 570

# DIFFUSION POTENTIALS IN MODELS AND IN LIVING CELLS

By W J V OSTERHOUT

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It is important to learn what fundamental properties of protoplasm are responsible for its electrical behavior. Progress in this field evidently depends on advances in physical chemistry and their use in biology.

Experiments on cells which are especially suitable for such studies show that they possess the properties of an aqueous system covered by a thin layer of non-aqueous material which is the chief seat of the electrical potentials. This material is present in exceedingly small amounts so that we can hardly hope to obtain enough for analysis. Failing this we may try to find models which act like the living cell. Much has been learned in this way.

A useful substance for this purpose is guaiacol which acts like certain protoplasmic surfaces in various ways, such as the following<sup>1</sup>

1 It allows water to pass freely; it admits inorganic electrolytes and to a still greater extent certain "lipoid-soluble" substances.

2 It is more permeable to potassium salts than to sodium salts and more permeable to chlorides than to sulfates.

3 When it is shaken with 0.01 M NaCl and placed in a U tube with aqueous 0.1 M NaCl on one side and aqueous 0.01 M NaCl on the other the dilute solution is electrically positive in the external circuit. This indicates that the mobility of  $\text{Na}^+$  (i.e.,  $u_{\text{Na}}$ ) is greater than that of  $\text{Cl}^-$  (i.e.,  $v_{\text{Cl}}$ ). This applies also to KCl and to the guaiaculates of sodium and potassium (which will be called for convenience KG and NaG).

4 When aqueous 0.1 M KCl is placed on one side of guaiacol (previously shaken with 0.1 M NaCl) and aqueous 0.1 M NaCl is placed on the other the KCl is negative in the external circuit ("potassium effect"). This indicates that  $u_{\text{K}}$  is greater than  $u_{\text{Na}}$ . This applies also to KG and NaG.

The study of guaiacol has especial interest because the role of diffusion potentials can be determined with considerable precision since Shedlovsky and Uhlig,<sup>2</sup> with the aid of the moving boundary measurements in guaiacol made by Longworth, have determined the mobilities in guaiacol of  $\text{K}^+$ ,  $\text{Na}^+$ , and the guaiacol ion together with dissociation constants and activities.

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Using these data we can predict diffusion potentials in cells of the type

Calomel electrode	Aqueous KG concentrated	Guaiacol	Aqueous KG dilute	Calomel electrode
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The calculations agree so well with the observed values that we may conclude that the latter are due to diffusion potentials

This is important in its bearing on the study of bioelectric behavior. Since the equations for diffusion potentials can be used in dealing with guaiacol there is reason to suppose that they may also be employed for *Nutella* and for other cells whose behavior resembles that of guaiacol. Hence we may calculate relative mobilities of ions and partition coefficients in the non-aqueous layer which covers the surface of the protoplasm and determine the effects of metabolism and applied reagents on ionic mobilities and on partition coefficients. This provides a method of studying protoplasmic behavior which is decidedly promising.

As an example of the situation in models we may consider the following: Guaiacol shaken at 25°C with aqueous 0.014 M KG until equilibrium was attained was placed in contact with aqueous 0.14 M KG on one side and with aqueous 0.014 M KG on the other. We may assume that the situation resembles that shown<sup>3</sup> in Scheme 1.

Aqueous		Guaiacol			Aqueous	
A	A'	B'	B	B''	C'	C
0.14 M	0.14 M	0.062 M	0.00094 M	KG	0.014 M	KG
KG	KG	KG				
P <sub>2</sub>	P <sub>3</sub>	P <sub>1</sub>			P <sub>4</sub>	

SCHEME 1

Here A' and B' represent exceedingly thin layers on each side of the phase boundary. We make the usual assumption that they at once come into equilibrium with each other. This also applies to B'' and C'.

We may suppose that when the guaiacol is placed in contact with 0.14 M KG there is a movement of KG from A' to B' making the concentration of KG in B' approximately 0.062 M. The amount of KG moving in this way is very small and it is quickly replaced in A' because KG in the aqueous phase diffuses up to the boundary much faster than it diffuses in the guaiacol phase since the viscosity of the latter is about 7 times as great as that of the aqueous phase.<sup>4</sup>

<sup>3</sup> Guaiacol in equilibrium with aqueous 0.14 M KG at 25°C contains 0.062 M KG. In equilibrium with aqueous 0.014 M KG it contains 0.00094 M KG.

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Hence any diffusion potential at  $P_2$  may be neglected on account of the very small magnitude of the concentration gradient in that region

The observed potential of the chain is therefore  $P_1 + P_2 + P_4$  where  $P_2$  and  $P_4$  are phase boundary potentials. If we compute the value of  $P_1$  and subtract it from the observed total value we can estimate the value of  $P_2 + P_4$

We may assume that at  $P_1$  we have a diffusion potential between 0.062 M KG and 0.00094 M KG in the guaiacol phase. To compute this we may employ the usual equation<sup>1</sup>

$$P_1 = \frac{RT}{F} (2t_K - 1) \ln \frac{a_1}{a_2} \quad (1)$$

where  $a_1$  and  $a_2$  are the mean ionic activities of KG in guaiacol and  $t_K$  is the transference number of  $K^+$  in guaiacol.

The values of  $t_K$  and  $t_{Na}$  were determined by Longworth as 0.57 and 0.54 from moving boundary measurements in guaiacol.<sup>2</sup> According to Shedlovsky<sup>3</sup> the equivalent conductivity of KG in guaiacol at zero concentration, i.e.  $\Lambda_0(KG)$ , is 9.5 so that we have for the equivalent conductivity of  $G^-$  at zero concentration, i.e.  $\lambda_0 = 9.5(0.43) = 4.085$ . For NaG we have  $\Lambda_0(NaG) = 9.0$  and for  $\lambda_0 = 9.0(0.46) = 4.14$ . These values agree within the limits

partition coefficient is  $0.062 + 0.14 = 0.44$  so that KG does not pass as readily into the guaiacol as it would into an aqueous solution.

The situation can be illustrated by using a dye as the diffusing substance. For this purpose brilliant cresyl blue (National Aniline Company) was allowed to diffuse into 1 part of guaiacol plus 25 parts of chloroform. The partition coefficient of the dye depends on the pH of the aqueous solution when this is about 5.8 the partition coefficient approaches 0.44.

We use a V tube (not a U tube) placing at the bottom the non-aqueous mixture of guaiacol plus chloroform. Above this we place in the left hand arm an aqueous solution of buffer and in the right hand arm brilliant cresyl blue dissolved in 0.01 M phosphate buffer solution at pH 5.8. Then diffusion takes place without producing a clear zone in the column of dye at the boundary (some dye enters the non-aqueous phase but this does not produce enough color to be visible). But if the partition coefficient is high (as when pure guaiacol is used as the non-aqueous mixture) the dye passes into the non-aqueous mixture faster than it can be brought up by diffusion and convection in the aqueous phase and in consequence a clear zone appears in the aqueous column of dye in the region of the phase boundary.

In Scheme 1 the movement of water across the boundary is neglected. If water tends to move from B to A at the start it tends to move back again as KG enters the guaiacol phase since guaiacol takes up more water when it contains KG any movement of guaiacol may also be neglected since both aqueous phases are saturated with guaiacol.

<sup>1</sup> Cf. MacInnes D. A. Principles of electrochemistry New York. Reinhold Publishing Corporation, 1939, pp 225-232.

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<sup>4</sup> Shedlovsky T., *J Gen Physiol*, 1942-43 26, 287

of experimental error<sup>7</sup> The average of these two values for  $\lambda_{\sigma}$  is 4.11 which gives for  $\lambda_K$   $9.5 - 4.11 = 5.39$  and for  $\lambda_{Na}$   $9.0 - 4.11 = 4.89$  Hence we shall use for the transference number of  $K^+$ ,  $t_K = 5.39 - 9.5 = 0.567$  and of  $Na$ ,  $t_{Na} = 4.89 - 9.0 = 0.543$

Equation (1) may also be written (for  $25^\circ C$ )

$$P_1 = 59(2t_K - 1) \log \frac{C_1 \theta_1 \gamma_1}{C_2 \theta_2 \gamma_2} \quad (2)$$

where  $\theta$  is the fraction dissociated,  $\gamma$  is the mean ionic activity coefficient, and the subscripts 1 and 2 refer to the two solutions

We may also write

$$P_1 = 59 \frac{u_K - v_{\sigma}}{u_K + v_{\sigma}} \log \frac{C_1 \theta_1 \gamma_1}{C_2 \theta_2 \gamma_2} \quad (3)$$

where  $u_K$  and  $v_{\sigma}$  are ionic mobilities, so<sup>8</sup> that  $t_K = u_K - (u_K + v_{\sigma})$  and  $t_{\sigma} = v_{\sigma} - (u_K + v_{\sigma})$  hence we may write

$$2t_K - 1 = \frac{2u_K}{u_K + v_{\sigma}} - \frac{u_K + v_{\sigma}}{u_K + v_{\sigma}} = \frac{u_K - v_{\sigma}}{u_K + v_{\sigma}}$$

We also have

$$2t_K - 1 = \frac{\lambda_{(K)} - \lambda_{(\sigma)}}{\lambda_{(K)} + \lambda_{(\sigma)}}$$

In order to compute the values of  $\theta$  and  $\gamma$  we determine the equivalent conductivity,  $\Lambda$ , at  $25^\circ C$  (see Fig. 1) According to Shedlovsky<sup>9</sup> we may write

$$\theta = \frac{\Lambda F}{\Lambda_0}$$

where  $\Lambda_0$  is the limiting equivalent conductivity at zero concentration (this is 9.5 for KG and 9.0 for NaG),<sup>6</sup>  $F = 1 + z + (z^2 - 2)$  and

$$z = \frac{\alpha \Lambda_0 + \beta}{\Lambda_0^{\frac{1}{2}}} \sqrt{C\Lambda}$$

where  $C$  is concentration in guaiacol For guaiacol at  $25^\circ C$  saturated with water Shedlovsky<sup>2</sup> gives the following values  $\alpha = 2.93$  and  $\beta = 19.36$  Hence for KG we have  $z = 1.611 \sqrt{C\Lambda}$  and for NaG we have  $z = 1.694 \sqrt{C\Lambda}$

<sup>7</sup> MacInnes,<sup>5</sup> p. 332 In applying these results we assume that  $t_K$  is constant which is approximately correct since the nearer  $t_K$  is to 0.5 the less it changes as concentration changes We also assume that  $\gamma$  is the same for both ions

<sup>8</sup> MacInnes,<sup>5</sup> pp. 59, 60 For univalent electrolytes we have for the equivalent conductivity  $\Lambda = \lambda^+ + \lambda^- = F a (u + v)$ , where  $F$  is the Faraday,  $a$  is the fraction dissociated,  $\lambda^+$  is the conductivity of the cation and  $\lambda^-$  of the anion

For  $0.062 \text{ M KG}$  at  $25^\circ\text{C}$   $\Lambda = 0.404$  and  $\theta = 0.0548$ , for  $0.00094 \text{ M KG}$   $\Lambda = 1.85$  and  $\theta = 0.208$

We may write according to Shedlovsky<sup>4</sup>

$$-\log \gamma = \frac{6.52\sqrt{C\theta}}{10 + 61\sqrt{C\theta}}$$

where  $\gamma$  is the mean ionic activity coefficient. We thus obtain for  $0.062 \text{ M KG}$  in guaiacol at  $25^\circ\text{C}$   $\gamma = 0.525$  and for  $0.00094 \text{ M}$   $\gamma = 0.824$ .

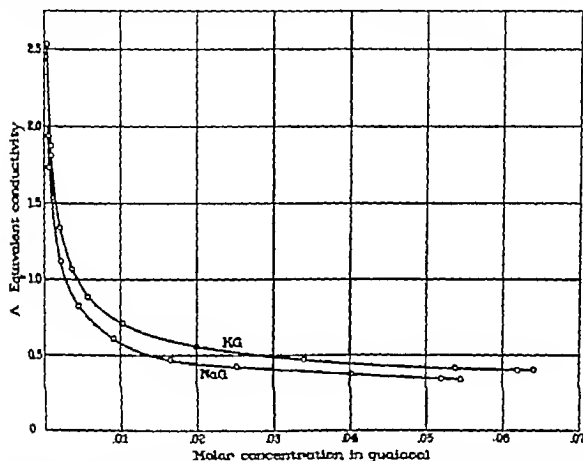


FIG 1 Curves showing the equivalent conductivity of potassium guaiacolate, KG and of sodium guaiacolate, NaG, at  $25^\circ\text{C}$  in guaiacol saturated with water (at each concentration the guaiacol was shaken with the appropriate aqueous solution until equilibrium resulted)

Inserting these values in equation (2) we have

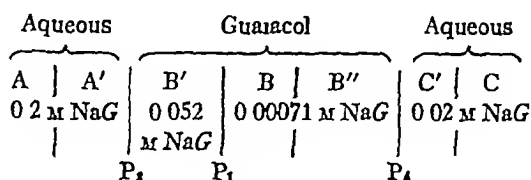
$$\begin{aligned} P_1 &= 59[2(0.567) - 1] \log \frac{0.062(0.0548)0.525}{0.00094(0.208)0.824} \\ &= 59(0.134) \log \frac{0.00178}{0.000161} \\ &= 59(0.134) \log 11.1 \\ &= 8.3 \text{ mv} \end{aligned}$$

The observed value is  $8 \pm 0.2$  (12 observations)

<sup>4</sup> These values apply to guaiacol in equilibrium with aqueous  $0.14 \text{ M}$  and  $0.014 \text{ M KG}$  respectively

It is of interest to note the difference between the concentration ratio  $0.062 - 0.00094 = 66$  and the ionic activity ratio  $0.00178 - 0.000161 = 11.1$ . The latter does not differ much from the ratio of aqueous concentrations, *i.e.*,  $0.14 - 0.014 = 10$ . Hence if we did not know the activities in guaiacol we should not be greatly in error in taking the ratio of aqueous concentrations (see p. 299).

To measure the concentration effect of NaG the same method was used. The dilute aqueous solution was  $0.02\text{ M NaG}$  which was shaken with the guaiacol, giving  $0.00071\text{ M NaG}$  in the guaiacol phase. At the other side was placed an aqueous solution of  $0.20\text{ M NaG}$ , giving  $0.052\text{ M NaG}$  in  $B'$  as shown in Scheme 2.



SCHEME 2

In the guaiacol phase we have the following values for  $0.052\text{ M NaG}$   $\Lambda = 0.346$ ,  $\theta = 0.0482$ , and  $\gamma = 0.562$ , for  $0.00071\text{ M NaG}$   $\Lambda = 1.93$ ,  $\theta = 0.228$ , and  $\gamma = 0.838$ .

Accordingly we have for the diffusion potential  $P_1$

$$\begin{aligned}
 P_1 &= 59(2[0.543] - 1) \log \frac{0.052(0.0482)0.562}{0.00071(0.228)0.838} \\
 &= 59(0.086) \log \frac{0.00141}{0.000136} \\
 &= 59(0.086) \log 10.4 \\
 &= 5.2\text{ mv}
 \end{aligned}$$

The observed value is  $5 \pm 0.1\text{ mv}$  (8 observations).

It is interesting to note the difference between the ratio of concentrations  $0.052 - 0.00071 = 73$  and that of the mean ionic activities  $0.00141 - 0.000136 = 10.4$ . The situation resembles that with KG where the corresponding values are 66 and 11.1 respectively. In this connection the following may be considered.

As long as the properties of the two phases remain unaltered the partition coefficient of the ionized portion of a solute is a constant when defined as

$$S_i = \frac{[C_i]}{[C'_i]}$$

where  $[C_i]$  refers to the non-aqueous and  $[C'_i]$  to the aqueous phase and the brackets denote activities. We have also  $[C_i]^2 = K[C_u]$  where  $K$  is the dis-

<sup>10</sup> Guaiacol shaken with aqueous  $0.2\text{ M NaG}$  at  $25^\circ\text{C}$  contains  $0.052\text{ M NaG}$ .

sociation constant and  $[C_u]$  is the activity of the unionized portion in the non aqueous phase. Hence we may write

$$S_i = \frac{\sqrt{K[C_u]}}{\sqrt{K'[C]}}$$

The partition coefficient for the unionized portion is a constant when defined as

$$S = \frac{[C_u]}{[C]}$$

Substituting this we obtain

$$S_i = \sqrt{\frac{KS}{K'}}$$

This treatment, due to Shedlovsky (personal communication), we may illustrate by the following in which hypothetical values are assumed in order to make a consistent scheme \*

Aqueous						S	Non-aqueous						S <sub>i</sub>	S
$\frac{C' = C_i + C_u}{C_i + C_u}$	$C_i$	$\gamma$	$[C_i]$	$K'$	$[C_u]$		$[C_u]$	$K$	$[C_i]$	$\gamma$	$C_i$	$\frac{C' = C_i + C_u}{C_i + C_u}$		
0.0012	0.0011	0.95	0.001	0.01	0.0001	4	0.0004	0.0001	0.0002	0.9	0.00022	0.00062	0.2	0.52
0.021	0.011	0.90	0.01	0.01	0.01	4	0.04	0.0001	0.002	0.68	0.003	0.043	0.2	0

\* For a simpler scheme see Osterhout, W J V *Biol. Rev.* 1931, 6, 400.

Here  $\gamma'$  is the ionic activity coefficient in the aqueous and  $\gamma$  that in the non aqueous phase (it is assumed that  $\gamma = 1$  for the unionized portion in both phases).  $S$  is the partition coefficient for the total concentration, i.e. for  $(C_i + C_u) + (C'_i + C'_u)$ .

It is evident that  $S_u$  is constant at 4 and  $S_i$  is constant at 0.2.  $S$  increases from  $0.00062 + 0.0012 = 0.52$  at the lower concentration, to  $0.043 + 0.021 = 2.0$  at the higher because  $K'$  is greater than  $K$  so that the ratio of undissociated to dissociated is greater in the non-aqueous phase and hence the concentration in the non-aqueous phase increases faster than in the aqueous phase.

When  $[C'_i]$  is multiplied by 10 we see that  $C'_i$  is multiplied approximately by 10,  $C'$  by 18,  $[C]$  by 10 and  $C$  by 69. This recalls the situation in guaiacol, as described earlier (p. 298).

Guaiacol in contact with aqueous solutions of KG or NaG takes up more water when the concentration of these substances increases and as the plait point<sup>11</sup> is approached the two phases become more and more alike so that the

<sup>11</sup> Cf. Osterhout W J V, and Murray, J W *J. Gen. Physiol.* 1939-40 23, 365



value of  $S_1$  approaches unity. Since the value of  $S_1$  in dilute solutions is much less than unity this taking up of water involves a rise in  $S_1$ . Hence the value of  $[C_1]_1 - [C_1]_2$  becomes progressively greater than that of  $[C_1']_1 - [C_1']_2$  (here the subscripts 1 and 2 refer to concentrated and dilute solutions respectively).

As previously stated (p 295) the observed potentials are theoretically equal to  $P_1 + P_3 + P_4$  and any excess of the observed values over the values calculated for  $P_1$  might be regarded as due to  $P_3 + P_4$ , i.e., to the phase boundary potentials. Since there is no excess, as shown in Table I, there is no reason to think that phase boundary potentials make any contribution to the observed values.

TABLE I  
Concentration Effects of KG and NaG

Concentrations in aqueous phase	Concentrations in non aqueous phase	Potential	
		Observed value	Calculated value of $P_1$ (diffusion potential)
0.14 M vs 0.014 M KG	0.062 M vs 0.00094 M KG	8	8.3
0.2 M vs 0.02 M NaG	0.052 M vs 0.00071 M NaG	5	5.2

#### EXPERIMENTAL

Guaiacol (Kahlbaum's c.p. crystallized or in some cases Eastman Kodak) was redistilled as described by Shedlovsky.<sup>2</sup> Owing to supercooling the guaiacol remained liquid at 25°C at which temperature all the measurements were made.

The solutions of KG and NaG were prepared by shaking guaiacol with aqueous KOH or NaOH free from carbonates. The concentrations of KG and NaG in the guaiacol were determined by shaking the guaiacol with water and titrating the aqueous solution while in contact with the guaiacol, using methyl red as an indicator (the endpoint was pH 5.0). A correction was made for the guaiacol dissolved in the aqueous phase.

On standing in contact with air these solutions acquired a color which deepened with time and the p.d. measurements became less reliable. It was therefore necessary to use freshly made solutions (in some cases solutions stored under nitrogen and free from color were employed).

Conductivity measurements in guaiacol nearly saturated with water were made following in general the method of Shedlovsky<sup>2</sup> (but using a Washburn type cell with a cell constant of 0.03741). In addition a series of measurements was made on guaiacol solutions which had been shaken with aqueous solutions of KG or NaG until equilibrium was attained (these are designated as "saturated with water" (Fig. 1)).

The measurements of potential were made with a Compton electrometer (Cambridge Instrument Co.) used as a null instrument. The solutions were placed in a

grounded wire cage and the wires connecting with the electrometer were shielded microphone cable.

The choice of electrodes is important<sup>12</sup> After experimenting with various kinds the choice fell upon the arrangement shown in Fig 2 The gualacol was contained in a breaker into which dipped 4 tubes filled with aqueous solution.<sup>13</sup> Thus in measuring the concentration effect of KG two tubes A and B, were filled with a dilute aqueous solution of KG and the other two with a more concentrated aqueous solution

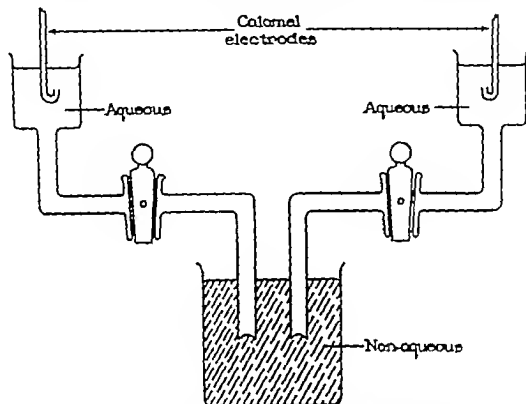


FIG. 2 To make liquid junctions between the gualacol and aqueous solutions the latter are placed in tubes which dip into the gualacol as shown The path traversed by the electric current in the gualacol has a large cross-section with no opportunity for short-circuiting by continuous aqueous films adhering to the glass Four tubes are employed (only two are shown) they are connected in turn by means of calomel electrodes to a Compton electrometer

of KG All of these were allowed to dip simultaneously into the gualacol and were connected in turn (with stopcocks closed) to the electrometer through calomel electrodes (filled with 3.5 M KCl) When the calomel electrodes were in proper condition the potential between A and B or between C and D did not exceed 1 or 2 mv and for this a correction could be applied. Under these conditions a close agreement of the potentials between A and C A and D B and C, and B and D was regarded as indicating a satisfactory state of affairs

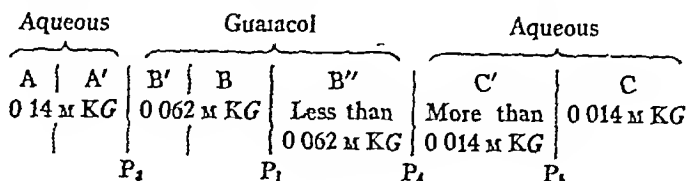
Under these conditions there was very little change of the values during the first hour (after this no readings were taken) except in the case of KG or NaG when the

<sup>12</sup> Stopcocks in gualacol should be avoided.

<sup>13</sup> The internal diameter of these tubes was 5 mm No stopcock grease was used

first reading was usually high, as explained later (p 304) If a change occurred in measuring concentration effects the measurement was rejected

Let us now turn to a different type of experiment in which the guaiacol is shaken at the start with the more concentrated aqueous solution Here the diffusion potential  $P_6$  in the dilute aqueous solution becomes important We may picture the situation as in Scheme 3



SCHEME 3

The maximum value possible for  $P_1$  would exist if B contained 0.062 M KG and B'', in equilibrium with C', contained 0.00094 M KG This maximum value would be calculated as 8.3 mv as described above (p 297)

The maximum possible value for  $P_6$  would result if C' contained 0.14 M KG, in equilibrium with 0.062 M KG in the guaiacol, and C contained 0.014 M KG We should then have<sup>14</sup> for  $P_6$  (assuming that concentrations in the aqueous solution are equal to activities)

$$P_6 = 59 \frac{\lambda_{(K)} - \lambda_{(G)}}{\lambda_{(K)} + \lambda_{(G)}} \log \frac{0.14}{0.014}$$

$$P_6 = 59 \frac{74 - 30}{74 + 30} \log \frac{0.14}{0.014}$$

$$= 25.0 \text{ mv}$$

Here the dissociation in the aqueous phase is regarded as complete

A measurement was made with the aqueous<sup>15</sup> solutions in direct contact (no guaiacol phase present) This gave  $23 \pm 0.4$  mv (8 observations)

Adding these maximum values we get  $P_1 + P_6 = 8.3 + 23 = 31.3$  mv This is, of course, larger than any value which could be realized in practice for the concentration of KG in C' would fall off rapidly since KG would diffuse away from C' into C much faster than it diffused up to C' in the guaiacol owing to the greater viscosity of the latter The observed value is  $19 \pm 0.6$  (12 observations)

As would be expected, we find that when we employ KCl in place of KG for the concentration effect we can neglect  $P_6$  since its value is very small in all cases Hence the observed value for the concentration effect of KCl is prac-

<sup>14</sup> The value of  $\lambda_K$  at 25°C is taken as 74 That of  $\lambda_G$  is taken as 4.11 (7.2) = 30 since the viscosity of guaiacol is 7.2 times that of water (cf Shedlovsky, T, and Uhlig, H H, *J Gen Physiol*, 1933-34, 17, 549)

<sup>15</sup> The aqueous solutions were saturated with guaiacol

tically the same whether we have a situation like that in Scheme 1 (p 294) or like that in Scheme 3

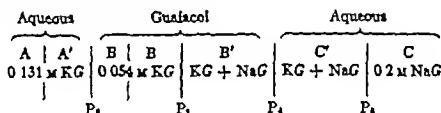
Similar considerations apply to NaG. The maximum value for  $P_1$  would be 5.2 mv, as already noted (p 298). For the maximum value of  $P_2$  we have<sup>16</sup>

$$P_2 = 59 \frac{50 - 30}{50 + 30} \log \frac{0.2}{0.02} \\ = 14.8 \text{ mv}$$

An actual test of the diffusion potential of 0.2 M NaG against 0.02 M NaG in water (no guaiacol phase present) gave  $17 \pm 0.1$  mv (4 observations)

Accordingly we have for the sum of the maximum values  $5.2 + 17 = 22.2$ . The observed value is  $15 \pm 0.03$  (4 observations) which is, as expected, much less

Turning now to the effect<sup>17</sup> of KG vs. NaG (potassium effect) we find it advantageous for purposes of computation to set up a chain in which the concentrations of the two salts in the guaiacol phase are approximately equal. For this purpose guaiacol was shaken with 0.131 M KG and then placed in contact with 0.2 M aqueous NaG, as in Scheme 4



SCHEME 4

The maximum value for  $P_1$  would occur if B contained 0.054 M KG (with no NaG) and B' contained<sup>18</sup> 0.052 M NaG (with no KG). This value cannot be accurately computed. We may attempt an approximation by assuming that in the guaiacol concentrations are equal to activities and that the degree of dissociation is the same for KG and NaG<sup>19</sup>. If we neglect the difference in

<sup>16</sup> The value of  $\lambda_{Na}$  in water at 25°C. is taken as 50 and that of  $\lambda_{Cl}$  as 4.11 (7.2) = 30 since the viscosity of guaiacol is 7.2 times that of water

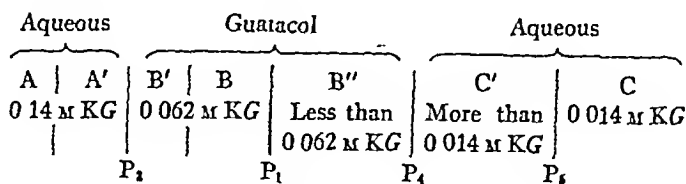
<sup>17</sup> It may be noted that in the cases previously treated where different concentrations of the same salt were in contact it makes no difference theoretically whether the diffusion boundary is sharp or diffuse. This does not apply when different salts are in contact. Cf. MacInnes D. A. <sup>5</sup> chapter 8 and p 224

<sup>18</sup> Guaiacol shaken with aqueous 0.131 M KG at 25°C contains 0.054 M KG shaken with 0.2 M NaG it contains 0.052 M NaG

<sup>19</sup> The degree of dissociation of KG and of NaG in guaiacol does not differ much (see footnote 6). The error involved in the assumption that concentrations in guaiacol are equal to activities is approximately the same for KG and NaG so that the resulting computation is not as far off as would otherwise be the case. It is lessened by the fact that only ratios are involved

first reading was usually high, as explained later (p 304) If a change occurred in measuring concentration effects the measurement was rejected

Let us now turn to a different type of experiment in which the guaiacol is shaken at the start with the more concentrated aqueous solution Here the diffusion potential  $P_5$  in the dilute aqueous solution becomes important We may picture the situation as in Scheme 3



SCHEME 3

The maximum value possible for  $P_1$  would exist if B contained 0.062 M KG and B'', in equilibrium with C', contained 0.00094 M KG This maximum value would be calculated as 8.3 mv as described above (p 297)

The maximum possible value for  $P_5$  would result if C' contained 0.14 M KG, in equilibrium with 0.062 M KG in the guaiacol, and C contained 0.014 M KG We should then have<sup>14</sup> for  $P_5$  (assuming that concentrations in the aqueous solution are equal to activities)

$$P_5 = 59 \frac{\lambda_{(K)} - \lambda_{(G)}}{\lambda_{(K)} + \lambda_{(G)}} \log \frac{0.14}{0.014}$$

$$P_5 = 59 \frac{74 - 30}{74 + 30} \log \frac{0.14}{0.014}$$

$$= 25.0 \text{ mv}$$

Here the dissociation in the aqueous phase is regarded as complete

A measurement was made with the aqueous<sup>15</sup> solutions in direct contact (no guaiacol phase present) This gave  $23 \pm 0.4$  mv (8 observations)

Adding these maximum values we get  $P_1 + P_5 = 8.3 + 23 = 31.3$  mv This is, of course, larger than any value which could be realized in practice for the concentration of KG in C' would fall off rapidly since KG would diffuse away from C' into C much faster than it diffused up to C' in the guaiacol owing to the greater viscosity of the latter The observed value is  $19 \pm 0.6$  (12 observations)

As would be expected, we find that when we employ KCl in place of KG for the concentration effect we can neglect  $P_5$  since its value is very small in all cases Hence the observed value for the concentration effect of KCl is prac-

<sup>14</sup> The value of  $\lambda_K$  at 25°C is taken as 74 That of  $\lambda_G$  is taken as 4.11 (7.2) = 30 since the viscosity of guaiacol is 7.2 times that of water (cf Shedlovsky, T, and Uhlig, H. H., *J. Gen. Physiol.*, 1933-34, **17**, 549)

<sup>15</sup> The aqueous solutions were saturated with guaiacol

Experiments with living cells indicate<sup>22</sup> that diffusion potentials play the chief rôle in their electrical behavior. Here we use a different method of calculation. Since we do not know the mobilities or activities in the non-aqueous protoplasmic surface layer we cannot employ them to calculate potentials. We must reverse the process and calculate ionic mobilities from the observed potentials. It is of interest to see how closely we approximate the true values when we use this method with guaiacol.

To determine relative mobilities from the concentration effect we may put  $v_o$  equal to unity and designate it as  $\bar{v}_o$ . We then have for the concentration effect of KG (p. 297), putting  $u_K + v_o = a_K$ ,

$$8 = 59 \frac{a_K - 1}{a_K + 1} \log \frac{a_1}{a_2}$$

With living cells there is a non-aqueous layer at the surface of the protoplasm and we may assume that the ratio of activities in this layer is the same as in the external aqueous solution (p. 306). Proceeding in the same way with guaiacol we may write (assuming that concentrations are equal to activities)

$$8 = 59 \frac{a_K - 1}{a_K + 1} \log \frac{0.14}{0.014}$$

whence  $a_K = 1.31$ . This means that  $u_K + v_o = 1.31$ . The actual value is the same as for  $\lambda_K + \lambda_o$ , i.e.  $5.39 + 4.11 = 1.31$  (p. 296).

In the same way from the concentration effect of 0.02 vs 0.20 M NaG which equals 5 (p. 298) we obtain  $a_{Na} = u_{Na} + v_o = 1.19$ . The actual value is  $4.89 + 4.11 = 1.19$  (p. 296).

In order to calculate  $t_K$ , the transference number of  $K^+$ , we have

$$t_K = \frac{a_K}{a_K + \bar{v}_o} = \frac{1.31}{1.31 + 1.0} = 0.567$$

The actual value of  $t_K$  as determined by Longworth is 0.57 (or as worked out above 0.567 (p. 296)).

The corresponding values for NaG are

$$t_N = \frac{a_N}{a_N + \bar{v}_o} = \frac{1.19}{1.19 + 1.0} = 0.543$$

<sup>22</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715; 1939-40, 23, 53; 171. Osterhout, W. J. V. and Hill, S. E., *J. Gen. Physiol.* 1938-39, 22, 139. Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.* 1937-38, 21, 541. *Proc. Nat. Acad. Sci.* 1938, 24, 312. Damon, E. B., *J. Gen. Physiol.*, 1932-33, 16, 375. Cowan, S. L., *Proc. Roy. Soc. London, Series B*, 1934, 115, 216. Erlanger, J., and Gasser, H. S., *Electrical signs of nervous activity*. The Eldridge Reeves Johnson Foundation for Medical Physics Lectures, Philadelphia, University of Pennsylvania Press, 1937, p. 134. Webb, D. A., and Young, J. Z., *J. Physiol.* 1940, 98, 299.

The actual value of  $t_{Na}$  as determined by Longworth is 0.54 or as worked out above 0.543 (p. 296)

It is evident that the method of calculation used for living cells gives satisfactory approximations to the true values when applied to a non-aqueous substance like guaiacol.<sup>23</sup>

This result depends upon two factors

1 The activity ratios Designating the activity of the ionized portion of a solute as  $[C_i]$  we may say that if the value of  $[C_i]$  in the aqueous phase increases tenfold it will also increase tenfold in the non-aqueous surface layer of the protoplasm. This implies that the partition coefficient of  $[C_i]$  is constant. As already stated (p. 298) this is true unless the non-aqueous phase changes its properties (e.g. by changing its content of water)

2 Diffusion potentials account for the observed P.D.'s The present paper shows that this is true for guaiacol and previous papers indicate that it applies to the cells most carefully studied, i.e., to *Nitella*, *Valonia*, and *Halocystis*. For example in *Nitella* the changes in P.D. due to changes in the concentration of KCl closely approach the values predicted by the equation for diffusion potentials.<sup>24,25</sup>

An extension of the method enables us to follow changes produced by reagents<sup>26</sup> or by metabolism<sup>27</sup> in mobilities and in partition coefficients.<sup>28</sup> For this purpose great accuracy is not needed since as a rule we are chiefly interested in qualitative results

<sup>23</sup> It does not follow that it would be equally useful with all non-aqueous substances

<sup>24</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1937-38, 21, 541. Also unpublished results with other salts. See also Blinks, L. R., The relation of bioelectric phenomena to ionic permeability and to metabolism in large plant cells, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, 8, 208.

<sup>25</sup> In some cases at least an entering cation may combine with an organic anion  $X^-$  in the non-aqueous surface layer (Jacques, A. G., *J. Gen. Physiol.*, 1939-40, 23, 41) but the anions in the external aqueous solution may also be important as shown by the change in P.D. when  $NO_3^-$  is substituted for  $Cl^-$  (Blinks, L. R., The relation of bioelectric phenomena to ionic permeability and to metabolism in large plant cells, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, 8, 204). The result would depend somewhat on the relative activity of  $X^-$ .

<sup>26</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 417, 1939-40, 23, 171. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938-39, 22, 139, *Proc. Nat. Acad. Sc.*, 1938, 24, 427.

<sup>27</sup> Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, 24, 312. Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, 23, 429.

<sup>28</sup> The use of Henderson's equation in determining partition coefficients makes the results less accurate.

As examples we may mention studies on *Nitella* which show that the mobilities and partition coefficients are by no means the same in winter as in summer<sup>27</sup> as a rule winter cells can be made to act like summer cells in these respects by leaching with distilled water which removes certain organic substances.

We also find that great changes are produced by reagents. For example, in *Nitella* certain mobilities and partition coefficients can be raised by guaiacol<sup>28</sup>. Guaiacol reverses the order of mobilities of  $K^+$  and  $Na^+$  in *Valonia*<sup>29</sup> so that instead of  $u_K > v_{Cl} > u_{Na}$  we have  $u_{Na} > v_{Cl} > u_K$  (thus showing that the surface is not a pore system)

Further studies in this field are very desirable.

It is a pleasure to thank Dr Theodore Shedlovsky for helpful suggestions and Mr Harry Bodner for the care and skill he has shown in making measurements.

#### SUMMARY

The behavior of guaiacol resembles that of certain protoplasmic surfaces to such an extent that it can be advantageously used in models designed to imitate certain aspects of protoplasmic behavior. In these models the electrical potentials appear to consist of diffusion potentials and this may be true of certain living cells.

In dealing with models we determine ionic mobilities and use these to predict potentials.

In studying living cells we measure potentials and from these calculate ionic mobilities. The question arises how far is this method justified. To test this we have treated guaiacol like a living cell, measuring potentials and from these estimating ionic mobilities.

The results justify the use of this method. This is of interest because the method is most useful in studying protoplasmic activity. In its extended form it enables us to follow changes in mobilities and in partition coefficients due to applied reagents and to metabolism.

<sup>27</sup> Osterhout, W J V. *J Gen Physiol*, 1939-40, 23, 171.

<sup>28</sup> Osterhout, W J V. *J Gen Physiol*, 1936-37, 20, 13.





# THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

## V THE INFLUENCE OF THE THICKNESS OF DRIED COLLODION MEMBRANES UPON THEIR ELECTROMOTIVE BEHAVIOR

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### I

The electrochemical properties of collodion membranes are known to vary greatly with the nature of the collodion preparation used, more acid preparations yielding membranes of higher electrochemical activity<sup>1-4</sup>. The assumption has always been made, tacitly or expressly, that the thickness of the membrane is of no influence. Thus, it has been generally assumed that the characteristic concentration potential<sup>5</sup> across dried collodion membranes must be independent of the thickness of the membranes.<sup>7</sup>

We became doubtful of the validity of this assumption. As was pointed out in a preceding communication<sup>8</sup> the concentration potentials across different membrane specimens frequently vary considerably, even if the membranes are prepared from the same collodion solution under conditions as nearly identical as possible. The following set of facts also needs to be considered. It has been demonstrated that the behavior of dried collodion membranes with solutions of strong electrolytes is due to (1) its micellar structural arrangement, that is, to the presence of interstices of larger than minimal (molecular) di-

<sup>1</sup> Michaelis L. and Perlzweig W. A. *J Gen Physiol* 1926-27, 10, 575

<sup>2</sup> Wilbrandt, W. *J Gen Physiol* 1935, 18, 933

<sup>3</sup> Sollner, K. and Abrams L. *J Gen Physiol*, 1940 24, 1

<sup>4</sup> Sollner K., Abrams L. and Carr C. W. *J Gen Physiol* 1941, 24, 467

<sup>5</sup> Sollner K. and Carr, C. W. *J Gen Physiol* 1942 26, 17

<sup>6</sup> The characteristic concentration potential is defined by Michaelis and collaborators as the potential which arises when a membrane is interposed between 0.01 M and 0.1 M potassium chloride solution. Michaelis, L., and Fujita, A., *Biochem Z*, Berlin, 1925 168, 28 1925 161, 47 1925 164, 23 Michaelis L., and Dokan, S., *Biochem Z* Berlin, 1925 162, 258 Michaelis, L., and Hayashi K., *Biochem Z*, Berlin 1926 173, 411 Michaelis, L. and Perlzweig, W. A., *J Gen Physiol*, 1926-27, 10, 575 Michaelis, L. McEllsworth R. and Weech, A. A., *J Gen Physiol* 1926-27, 10, 671 Michaelis, L. Weech A. A. and Yamaton, A., *J Gen Physiol* 1926-27 10, 685 Michaelis L., *Bull Nat Research Council*, No 69, 1929 119 *Kolloid Z* 1933 62, 2 and other publications.

<sup>7</sup> Weech A. A. and Michaelis, L. *J Gen Physiol* 1928 12, 221

mensions,<sup>5</sup> and (2) to the dissociable groups—carboxyl groups—present in some of the nitrocellulose molecules.<sup>4,8,9</sup> It was further shown (3) that the surface concentration of these “active” groups—as determined by base exchange experiments—can be very small, even in highly active collodion preparations, smaller actually than can be determined with the conventional chemical and physical methods.<sup>9</sup> Thus, there is some doubt as to whether there is a sufficient number of these active groups available to supply each of the interstices across a thin membrane with at least one active group.<sup>5,9</sup> It is obvious that the chance of this occurrence diminishes with decreasing thickness of the membrane.

In the foregoing discussion we have assumed that a single active group in an interstice across the thickness of the membrane is sufficient to cause the maximal effect, *i.e.*, complete blocking of the passage of ions of the same sign. This assumption is supported by all the available experimental data, and is tacitly made in all theoretical work.

The measurable membrane potential with each individual membrane is, of course, a mean of the potentials of all the possible pathways across the membrane, each pathway contributing to the measurable effect according to its electrical characteristics, pore potential, and resistance. In other words, the pores which do not contain a dissociable group short circuit the electrical effect set up by the pores containing one or several such groups. For membranes containing somewhat larger pores this general concept has been used to explain the mechanism of anomalous osmosis.<sup>10</sup> It is obvious that unduly large pores, and still more so, any cuts, holes, or cracks would influence the measurable effect very strongly by such short circuits.

In the experimental part we will discuss briefly the reasons why we feel certain that no larger mechanical defects are present even in thin collodion membranes and what evidence we have that unduly large pores (which could not be blocked by a single charge) do not seem to exist.

For reasons outlined above we decided to determine experimentally whether or not the concentration potential across dried collodion membranes is a function of their thickness. Any result is bound to cast considerable light on the interaction of geometrical and electrical factors in the constitution of the dried collodion membrane.

In addition a definitely positive decision is bound to contribute pertinent information concerning the relative merits of the continuous phase theory and

<sup>8</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, 25, 7.

<sup>9</sup> Sollner, K., Carr, C. W., and Abrams, I., *J. Gen. Physiol.*, 1942, 25, 411.

<sup>10</sup> Sollner, K., *Z. Electrochem.*, 1930, 36, 234, Sollner, K., and Grollman, A., *Z. Electrochem.*, 1932, 38, 274, Grollman, A., and Sollner, K., *Tr. Electrochem. Soc.*, 1932, 61, 477, 487, Sollner, K., *Kolloid-Z.*, 1933, 62, 31.

the micellar structural theory, a problem which was dealt with from another point of view in the preceding communication.<sup>11</sup>

The homogeneous phase theory would predict the concentration potential to be independent of thickness, unless special effects at the interfaces, for example orientation or film formation, came into play. In this case, which has not found adequate treatment in the literature, the interfacial layers would be different from the bulk phase, the whole interphase would be built up as interfacial layer/bulk phase/interfacial layer. Such a 'structural' arrangement of the "homogeneous" interphase could conceivably account for a dependence of the concentration potential upon the thickness of the membrane. However, if such conditions prevail, the homogeneous phase theory, as we are accustomed to think of it, would cease to be applicable.

A 'micellar structural' theory would, at least for certain favorable cases, lead to the prediction that increasing thickness of the membrane would bring about increasing ionic selectivity, and consequently increasing concentration potentials provided, as indicated above, that the thickness of the membranes be not great compared with the mean distance between two electrochemically active groups or spots within the membrane. In a highly schematic manner Figs 1 and 2 give two possible ways to visualize the thickness effect. We assume, in agreement with the best information available, that the charged spots in a narrow pore prevent the passage of ions of the same sign but allow the passage of oppositely charged ions.<sup>12</sup> Uncharged pores of proper dimensions allow the passage both of anions and cations. In the figures the membrane substance is indicated by striation, spots which prevent the passage of anions, due to a fixed negative charge (as is the case in collodion) are denoted by asterisks.

Figs 1a, 1b, and 1c show the simplest conceivable structure of a porous membrane. Fig 1a, representing a unit thickness of membrane, shows every other pore blocked for the passage of anions, the ratio of the free to the geometrically possible pathways thus being 0.5. If in a membrane twice as thick (Fig 1b) the charges are distributed at random, the number of pathways free for the permeation of anions would be  $0.5 \times 0.5 = 0.25$  or 25 per cent the ratio of

<sup>11</sup> For a brief statement of the problem, homogeneous phase theory *versus* micellar structural theory and for some pertinent literature, the reader is referred to our preceding communication (Sollner K., and Carr C. W. *J Gen Physiol*, 1942 26, 17.)

<sup>12</sup> The interstices in dried collodion membranes conform to this condition. This can readily be seen from the fact that dried collodion membranes on activation by oxidation become practically impermeable to anions, as evidenced by the nearly maximal concentration potentials across such membranes. (Sollner K., Abrams I. and Carr, C. W. *J Gen Physiol* 1941 25, 7; Sollner, K., and Carr, C. W. *J Gen Physiol* 1942 26, 17.)

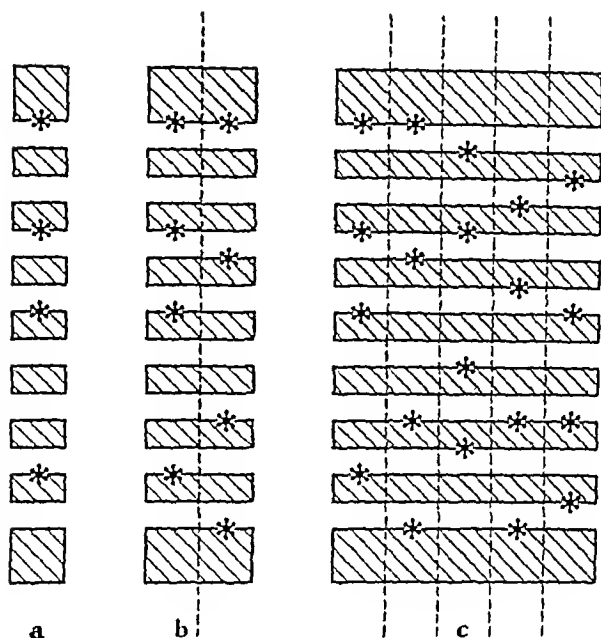


FIG 1

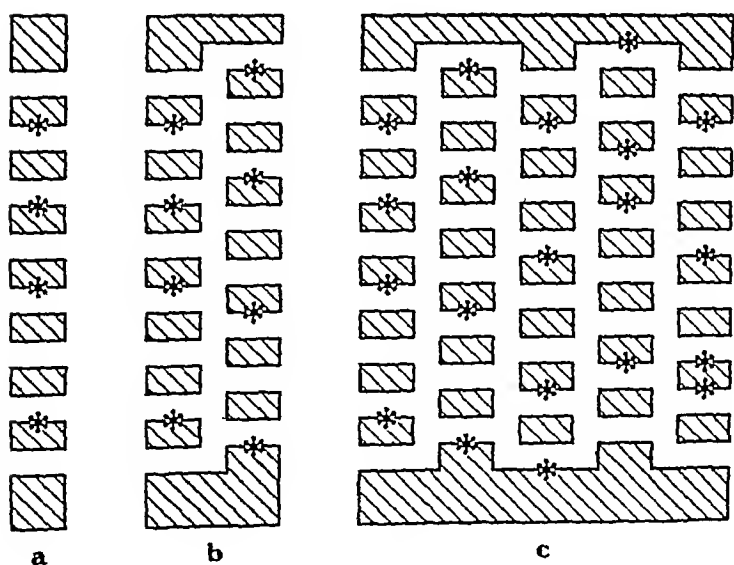


FIG 2

possible pathways to pathways available for anions thus decreases in a geometrical series with increasing thickness of the membrane, Fig 1c, e g represents a five-layer membrane, which gives (statistically) a ratio of  $1 \left(\frac{1}{2}\right)^5 \approx 1 \frac{1}{32}$

The potential across such membranes between the concentrations  $c_1$  and  $c_2$  in the case of a uni-univalent electrolyte can, according to Michaelis, be expressed as

$$(1) \quad E = \frac{U - V}{U + V} RT \ln \frac{c_1}{c_2}$$

$U$  and  $V$  being respectively the mean mobilities of all the cations and anions in the pores. However, this approach is too formal for our present purposes. Recently, it was shown experimentally that a certain number of anions are fixed permanently and immovably to the structure of the membrane, their mobility thus being zero.<sup>13,14,15,16,17,18</sup>

It is outside the scope of this paper to discuss all the ramifications of this general concept. Our considerations are actually based on certain simplified assumptions. Nevertheless, they are sufficiently accurate to yield at least a semiquantitative picture of the true situation. Readers interested in the theoretical aspects of the matter must be referred to the papers of Teorell,<sup>13</sup> and Meyer and Sievers<sup>14, 15</sup> which are based on a rigorous quantitative application of the above general concept.

If the fraction  $a$  of all possible pathways in the membrane is blocked for the movement of anions (but allows the passage of cations) and if  $u$  and  $v$  are the true mobilities of the movable cations and anions in the membrane,  $v_0$  being the mobility of the fixed anions, the Nernst equation can be written as follows

$$(2) \quad E = \frac{u - (1-a)v - av_0}{u + (1-a)v + av_0} RT \ln \frac{c_1}{c_2}$$

and since

$$(3) \quad \begin{aligned} v_0 &= 0 \\ E &= \frac{u - (1-a)v}{u + (1-a)v} RT \ln \frac{c_1}{c_2} \end{aligned}$$

In the case of a membrane with  $n$  unit layers the equation for the case discussed above becomes

$$(4) \quad E_n = \frac{u - (1-a)v}{u + (1-a)v} RT \ln \frac{c_1}{c_2}$$

since only the fraction  $(1-a)$  of all possible pathways is available to anions.

Experiments of Meyer and Sievers<sup>15</sup> indicate that the ratio of the mobilities of univalent anions and cations in a membrane of porous character does not

<sup>13</sup> Teorell, R. *Proc. Soc. Exp Biol and Med* 1935 33, 282

<sup>14</sup> Meyer K. H. and Sievers J. F. *Helv Chim Acta* 1936, 19, 649

<sup>15</sup> Meyer K. H., and Sievers, J. F. *Helv Chim Acta* 1936, 19, 665, 963

differ appreciably from the ratio in free solution. If we assume this to be correct we may introduce the conventional figures for  $u$  and  $v$  in our equations. For the case of potassium chloride  $u$  is equal to  $v$ . If we consider this case, and a concentration ratio of 10:1 and further, a selectivity factor  $a$  per unit layer of  $\frac{1}{2}$ , as assumed in Fig. 1, then a monolayer membrane would yield 18.7 mv, a membrane composed of two layers 33.6 mv, a five-layer membrane 52.6 mv, etc., if the thermodynamically possible maximum is assumed to be to 56 mv.

However, the kind of very simple behavior which we just discussed is by no means necessarily inherent in membranes which have a structure. Another possibility is shown in Fig. 2. Fig. 2a represents a unit layer of membrane, 50 per cent of the pores being blocked for anions ( $a = 0.5$ ). Fig. 2b shows a possible assembly of two such layers which allow a free cross connection of the pores between two adjacent membrane layers. If an electrolyte diffuses through such a membrane this space will soon be filled with electrolyte solution, the concentration of which,  $c_3$ , is somewhere between the concentrations  $c_1$  and  $c_2$  on the two sides of the membrane. The potential across the whole of the membrane  $E_{1,2}$  is the sum of the two single potentials across the two individual membrane layers.

$$(5) \quad E_{1,2} = E_{1,1} + E_{2,1}$$

$$(6) \quad E_{1,1} = \frac{u - (1-a)v}{u + (1-a)v} RT \ln \frac{c_1}{c_3}$$

and

$$(7) \quad E_{2,1} = \frac{u - (1-a)v}{u + (1-a)v} RT \ln \frac{c_3}{c_2}$$

Therefore

$$(8) \quad E_{1,2} = \frac{u - (1-a)v}{u + (1-a)v} RT \left( \ln \frac{c_1}{c_3} + \ln \frac{c_3}{c_2} \right) =$$

$$(9) \quad E_{1,2} = \frac{u - (1-a)v}{u + (1-a)v} RT \ln \frac{c_1}{c_2}$$

For a membrane of  $n$  layers thickness the equation becomes

$$(10) \quad E_{1,2} = \frac{u - (1-a)v}{u + (1-a)v} RT \left( \ln \frac{c_1}{c_a} + \ln \frac{c_a}{c_b} + \dots + \ln \frac{c_{(n-2)}}{c_{(n-1)}} + \ln \frac{c_{(n-1)}}{c_2} \right) \\ = \frac{u - (1-a)v}{u + (1-a)v} RT \ln \frac{c_1}{c_2}$$

Thus the potential across a membrane as sketched in Figs. 2b and 2c is independent of the number of unit layers (plus connecting spaces) from which

the membrane is built. We also see that the concentration potential is independent of the distribution of the concentration drop from  $c_1$  to  $c_2$  among the several layers of the membrane

Thus, we see that cross connections between different pores influence the behavior of membranes profoundly. According to the micellar structural theory therefore, the relative frequency of the blocking groups and branchings of the pores should be the paramount factor determining the electrochemical behavior of dried collodion membranes.

Many cases of a more complicated nature than those which were discussed are of course accessible to theoretical treatment. However, any further discussion seems superfluous at this point. The effects may lie anywhere between the two extremes represented by the two cases mentioned. No definite prediction as to the dependence of the concentration potential upon the membrane thickness can be made on the basis of the micellar structural theory, the latter is compatible with potentials which depend upon the membrane thickness as well as with potentials which are independent of it. However one can expect the appearance of the thickness effect with some degree of certainty merely from a consideration of the chance distribution of charges. One would anticipate this effect particularly for very thin membranes prepared from not too active collodion preparations.

It is obvious that the absolute permeability per square centimeter of membrane decreases in all cases as the membrane thickness increases, with electrolyte solutions this means increased ohmic resistance with increasing thickness. However, for the present we are not concerned with this obvious effect, but will consider and investigate only the magnitude of the electromotive forces which may be caused by different membrane thicknesses.

## II

The experiments which are necessary to test for the existence of the expected thickness effect are of an extremely simple nature: the electromotive properties, e.g. the characteristic concentration potential,<sup>6</sup> of dried membranes are determined. Several collodion preparations of different activity were used, since this factor could easily be of great importance. One could expect that even very thin membranes prepared from a highly active preparation might yield nearly maximum concentration potential values, in this case, of course, no significant increase with increasing thickness could be expected.

Membranes cast from the same collodion solution under supposedly identical conditions may yield concentration potentials which vary widely.<sup>2-4</sup> It therefore was necessary to cast a number of membranes from each solution in order to obtain significant mean values for the concentration potential.

Our experiments were carried out with bag-shaped membranes, since it is much easier to work with a large number of bag-shaped membranes than with



flat ones. Bag-shaped membranes have the additional advantage that rather thin membranes may be readily used.

Membranes of different thickness are most easily obtained by casting them from solutions of different concentration, the more concentrated solutions are more viscous and thus readily yield membranes of greater thickness, if one uses the conventional manual casting method.

This method is by no means perfect. Though the average thickness (see below) of different membrane specimens is remarkably constant in any given series, nevertheless, slightly thinner and thicker spots appear in each individual membrane. However, we want to stress the fact that a trained experimenter is able to produce membranes of great uniformity.<sup>16</sup>

Most of our membranes—many show bright interference colors when immersed in water—are too thin for accurate mechanical measurement of their thickness. We therefore determined their average thickness by area and weight determination in the dry state. The weight and area of several membranes of each kind were measured after carefully removing all unduly heavier parts which are frequently found near the open end of the membrane bags. The specific gravity of the dry membranes was taken as 1.6.

Here it may be asked what proof exists that the membranes, particularly the thinner ones, are intact. There is first, the method of Northrop,<sup>17</sup> who tested membranes by filling them with 2 M sodium chloride solution and immersing them in 1 M silver nitrate, in this way holes may readily be detected by the formation of a precipitate. Further, there is the fact that considerable concentration potentials are consistently obtained. Occasionally, but rarely, a membrane is found which gives a zero concentration potential, or nearly so. Such membranes can nearly always be shown to leak and are discarded. That the membranes may be oxidized to yield nearly maximum concentration potentials (regardless of the potential they give in the original unoxidized state) seems to indicate strongly that they are without holes or cracks. That the membranes may be handled and stored for considerable time without any change in the characteristic concentration potential also indicates the absence of any cracks or cuts which would tend to grow in course of time.

The collodion preparations used were Baker Collodion, USP and Mallinckrodt "Parlodion," two of the best commercial preparations, a sample of oxidized collodion of very high electrochemical activity, and finally, purified "Parlodion," the least active preparation known to us. The methods of preparing the latter two preparations were described in previous papers.<sup>8,9</sup> There one finds also data concerning their general properties and their electrochemical behavior. The batch of Mallinckrodt "Parlodion" used in the present work was significantly more active than batches used previously.

<sup>16</sup> E.g. Nakagawa, J., *Jap J Med Sc III Biophysics, Transactions and Abstracts*, 1937, 4, 297.

<sup>17</sup> Northrop, J. H., *J Gen Physiol*, 1928, 11, 233.

Solutions of 2, 4, 6, 8, and 10 per cent collodion in 75 per cent absolute ether and 25 per cent absolute alcohol were used. Appreciably more dilute solutions yield membranes which cannot be handled conveniently in the usual manner, whereas solutions containing more than 10 per cent collodion are—with the preparations used—too viscous to allow a satisfactory casting of membranes.

There is an additional method of preparing membranes of increasing thickness, namely, by superimposing several layers of collodion on top of each other, a new layer being added after the preceding one is dried completely. This method, however, suffers from the disadvantage that thin membranes (as prepared from dilute solutions) are largely dissolved on contact with the collodion solution added for the preparation of the next layer. With more concentrated solutions this effect is not so conspicuous, but there is a strong tendency for the membranes to loosen from the wall of the test tube in which they are cast, in this case irregular shrinkage sets in, thus causing the loss of many membranes.

The membranes were cast by hand in  $22 \times 85$  mm test tubes, dried for about 24 hours, and loosened in the usual way by the addition of distilled water. Next the membranes were fitted to glass rings for more secure and convenient handling. It was found to be immaterial whether the membranes were kept dry or immersed in distilled water prior to measuring the concentration potentials. The latter were measured in the usual manner, as described by Michaelis and collaborators.<sup>6</sup> Since the ohmic resistance of many of the membranes (the thick ones prepared from high grade collodion) is rather high, the volt scale of a commercial electron tube voltmeter (Leeds and Northrup glass electrode) was used as potentiometer in spite of its limited ( $\pm 1$  mv) accuracy. The potential values are easily and accurately reproducible with each membrane. On contact with the salt solution the final concentration potential is established nearly immediately within very few millivolts and reaches the final value in any case within a few minutes. All potential values given in the tables are final, reproducible values. Our membranes were, with very few exceptions, symmetrical within the limits of experimental error, the same potential being obtained whether the concentrated solution was in contact with the inside or outside of the membrane. The asymmetry of the few not completely symmetrical membranes was too small to influence our results to an appreciable extent.

Tables I and II present observations in representative series of our experiments. Each potential value represents one individual membrane. Fig. 3 summarizes the results graphically. All experimental values in Table I, Table II, and Fig. 3 are given as obtained at room temperature without correction. No membranes were left out of the tabulation except those which were manifestly damaged.

The mean of the concentration potential of each series of membranes, the magnitude which is of primary interest here, is given in bold face type at the bottom of the individual columns together with the standard deviation of the

TABLE I

*The Influence of the Thickness upon the Concentration Potential 0.1 M KCl/0.01 M KCl across Dried Collodion Membranes Membranes Prepared from Collodion Solutions of Different Concentration Potentials in Millivolts*  
*A Mallinckrodt Parlodion*

2 per cent (Average mem- brane thickness 2.7 $\mu$ )	4 per cent (Average mem- brane thickness 6.1 $\mu$ )	6 per cent (Average mem- brane thickness 8.9 $\mu$ )	8 per cent (Average mem- brane thickness 18 $\mu$ )	10 per cent (Average mem- brane thickness 27 $\mu$ )
<i>mv</i>	<i>mv</i>	<i>mv</i>	<i>mv</i>	<i>mv</i>
33	24	36	42	46
33	43	42	44	42
10	44	41	43	43
20	40	40	44	40
32	22	40	44	43
20	40	40	44	41
30	37	23	43	42
29	38	40	38	42
33	40	42	45	43
28	42	41	42	41
27	40	40	44	45
10	39	41	42	43
25	32	40	43	42
26	36	42	41	42
27	23	40	41	45
26	15	32	42	40
26	34	33	42	42
18	32	35	40	45
31	33	41	41	42
33	35	39	40	45
Mean 25.6 $\pm$ 1.6	Mean 34.6 $\pm$ 1.8	Mean 38.4 $\pm$ 1.0	Mean 42.3 $\pm$ 0.4	Mean 42.6 $\pm$ 0.4

*B Purified Mallinckrodt Parlodion*

2 per cent (Average membrane thickness 3.6 $\mu$ )	6 per cent (Average membrane thickness 12.0 $\mu$ )	10 per cent (Average membrane thickness 32.2 $\mu$ )
<i>mv</i>	<i>mv</i>	<i>mv</i>
24	31	37
21	31	29
27	29	30
26	30	27
15	29	30
24	30	30
17	32	28
	32	35
	33	40
	31	
	32	
Mean 22.0 $\pm$ 1.7	Mean 30.9 $\pm$ 0.4	Mean 31.8 $\pm$ 1.5

TABLE I—Continued  
C Baker Collodion U.S.P

2 per cent (Average mem- brane thickness 2.7 $\mu$ )	4 per cent (Average mem- brane thickness 4.4 $\mu$ )	6 per cent (Average mem- brane thickness 12.0 $\mu$ )	8 per cent (Average mem- brane thickness 21.5 $\mu$ )	10 per cent (Average mem- brane thickness 29.9 $\mu$ )
mv	mv	mv	mv	mv
25	29	42	49	45
22	34	42	42	43
27	32	41	46	46
23	27	40	38	45
24	26	32	39	45
24	35	37	40	43
19	25	42	40	44
	25	41	40	41
Mean	Mean	Mean	Mean	Mean
23.4 $\pm$ 1.1	29.1 $\pm$ 1.4	39.6 $\pm$ 1.2	41.7 $\pm$ 1.3	44.0 $\pm$ 0.6

D Oxidized Collodion

2 per cent (Average membrane thickness 2.7 $\mu$ )	4 per cent (Average membrane thickness 5.9 $\mu$ )	6 per cent (Average membrane thickness 18.2 $\mu$ )
mv	mv	mv
44	52	54
49	51	54
47	49	53
50	53	55
44	44	56
47	53	55
44	47	55
	53	54
		54
Mean	Mean	Mean
46.4 $\pm$ 1.0	50.2 $\pm$ 1.2	54.4 $\pm$ 0.3

TABLE II

The Influence of the Thickness upon the Concentration Potential 0.1 M KCl/0.01 M KCl across  
Dried Collodion Membranes Poly-layer Membranes Prepared from 8 Per Cent  
Solutions Concentration Potentials in Millivolts

A Purified Mallinckrodt Parlodion	B Baker collodion U.S.P	
4 layers (average membrane thickness 162 $\mu$ )	2 layers (average membrane thickness 79.7 $\mu$ )	4 layers (average membrane thickness 117 $\mu$ )
mv	mv	mv
31	43	47
29	43	45
33	40	47
38	43	45
29		
30		
Mean	Mean	Mean
31.7 $\pm$ 1.4	42.5 $\pm$ 0.8	46.0 $\pm$ 0.6

mean From the standard deviations of the means the high statistical significance of the differences in potential values of adjacent points on the ascending branches of the curves (Fig 3) can readily be inferred Thus, in the case of Mallinckrodt Parlodion the probability is 5000 : 1 that the difference between the values pertaining to the 2.7 and 6.1  $\mu$  membranes is not due to chance, it is only 15 : 1 for the 6.1 and 8.9  $\mu$  membranes, corresponding to the relatively small difference in the membrane thickness The difference between the 8.9 and 18  $\mu$  membranes is not due to chance, with a probability of better than 3000 : 1 The difference between the 18 and 27  $\mu$  membranes, however, has a chance less than 1 : 1 to be significant The significance of the thickness effect

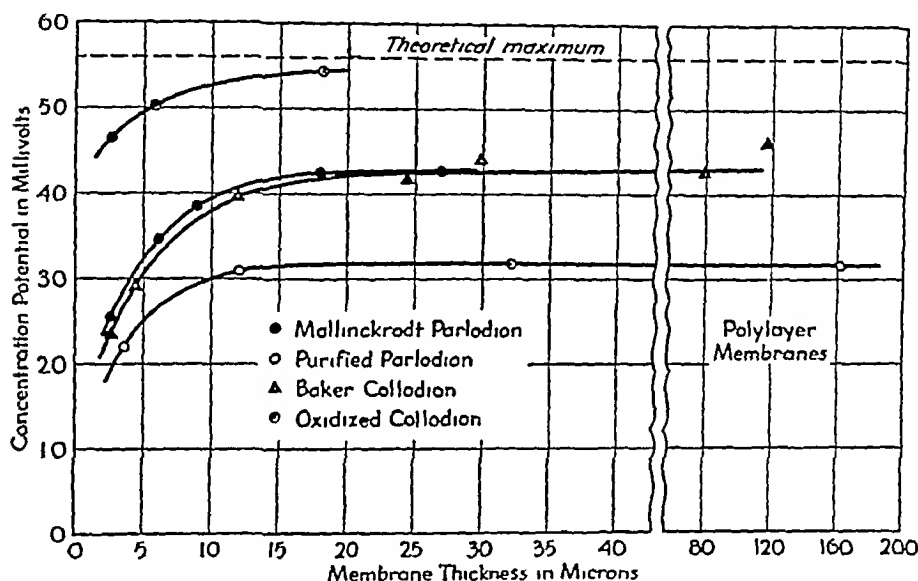


FIG 3

with the other brands of collodion is similar to that given for Mallinckrodt Parlodion

### III

The general results of the experiments presented in Tables I and II can be read conveniently from Fig 3 and may be summarized as follows —

1 The concentration potential is a function of the membrane thickness with all the collodion preparations which were investigated

2 With electrochemically *active* collodion nearly maximum values were obtained with membranes of less than 0.01 mm thickness, the theoretically possible maximum being reached within 2 mv with membranes of about 0.02 mm thickness

3 With rather *inactive* collodion preparations the potentials increase from low values in the case of the thinner membranes to medium values with thicker (0.02–0.03 mm) membranes Still thicker membranes do not yield appreci-

ably higher concentration potentials, actually, there is no indication that the theoretically possible maximum value could be obtained with membranes of any thickness

The former assumption that the concentration potential is independent of membrane thickness must be abandoned. Parenthetically we may add that this conception had its origin in all probability in the preferential use of active collodion for membrane work and also in the nearly universal use of membranes of appreciably greater thickness than those used for the foregoing experiments

If we recall the discussion in the first half of this paper we further come to the conclusion that the homogeneous phase theory cannot be applied to dried collodion membranes, as this theory predicts independence of concentration potential and membrane thickness. The experimental results are compatible in a general way with the micellar-structural theory. However, neither of the two simple cases discussed in the first half of this paper fits the experimental results adequately over the whole range of investigated membrane thicknesses

The first of the suggested simple structural possibilities predicts an increase in the concentration potential and finally an approach to the maximum possible potential with increasing membrane thickness according to equation 4. The ascending branches of the curves agree in a qualitative way with this concept, however, we see that this maximum value is not reached as a rule. Rather, a constant potential value, different from the thermodynamically possible maximum is reached at a certain thickness of the membrane, this value does not seem to increase with still thicker membranes. Even if we consider only the ascending branches of the curves, it seems impossible to apply equation 4, whatever values for  $\alpha$  (and the unit thickness of membrane) are tried, the experimental curves are too flat. The horizontal part of the curves, on the other hand, would be consistent with the second of the above possibilities as expressed in equation 10, however, this equation does not account at all for the ascending branches of our curves.

Obviously, the two possibilities which were discussed at the beginning of this paper are too simple. Our problem therefore is to find a consistent and inherently probable way to explain the shape of our curves, not only for the experimentally accessible range, but also for the range of still thinner membranes. Our experimental curves have to be extrapolated to or at least near to the point of intersection of the thickness and of the potential axes.

Any useful explanation obviously needs to assume greater structural complexity of the membranes than the two suggested possibilities. It is rather suggestive to assume that the orientation and aggregation of the collodion molecules in the surface layers and near them is different from the situation in the interior of the membrane. To simplify the argument we assume in the following discussion that the two sides of the membrane are equivalent or at least analogous in their structural buildup, this, however, is by no means neces-

sarily true, as the forces acting upon the two sides of the membrane during the process of drying are not exactly identical. It seems probable that the molecular aggregation in the surface layers should be denser and more regular than in the interior of the membrane where these forces are not operative. Corresponding to this one would find in the surface layers a smaller specific permeability and greater ionic selectivity. According to this view, the membrane would be made up of qualitatively different layers, the surface layers would be mainly responsible for the electrical and general permeability properties of the membrane, the inner layers acting as a more indifferent supporting structure.

This picture agrees well with the experimental results. The steepness of the ascending branches of our curves in the region of the thinnest membranes indicates a very great specific ionic selectivity per unit thickness in this region in a structure conforming in some degree to the picture of Fig. 1. In the addi-

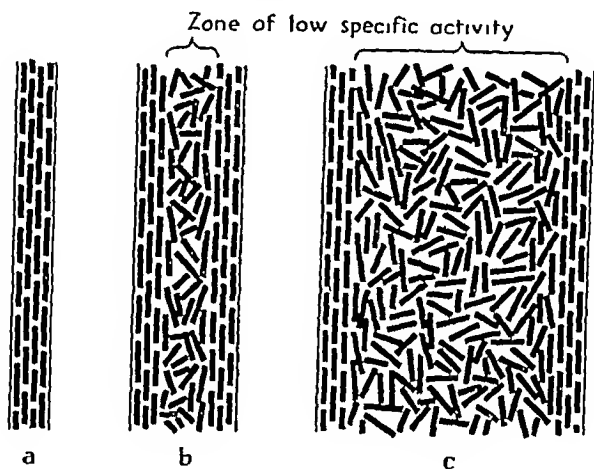


FIG. 4

tional layers of somewhat thicker membranes the selectivity becomes less, hence the rapid flattening out of our curves which is incompatible with equation 4. If the collodion does not happen to be very active, the aggregate selectivity of these layers on the two sides of the membrane is not sufficient to cause maximum concentration potentials. The additional thickness of the membrane is composed of a structure of relatively low specific selectivity, probably due to a somewhat looser state of aggregation of the collodion molecules. These inner layers then would contribute practically nothing to the overall selectivity of the membrane, whatever their thickness may be. These layers thus conform to the picture sketched in Fig. 2.

According to the ideas outlined, membranes above a certain minimum thickness are built up of identical active structures with or without an inactive interlayer. The assumed relationship between a very thin membrane and two constant potential value membranes of different thickness is sketched in Figs. 4a, b, and c.

From this discussion it can be concluded that the experimental facts can readily be explained by the assumption of a structure which is a combination of the two simplest possible structures which were mentioned in the first part of this paper. The factual state of any given layer of a membrane probably is between the two extremes, a great variety of intermediate states occurring in each membrane. However, until further proof is available this picture must be considered as entirely hypothetical.

With polylayer membranes, as said before, each previous layer is partially dissolved when one adds another. Here, too, as it appears from the results, the two outermost layers of the membrane are probably responsible for the observed concentration potentials. However, at this time it seems superfluous or at least premature to discuss this possibly more complex case in greater detail.

Further discussion of the significance of the above results with regard to the problem of membrane structure in general must be postponed. Only after additional data concerning water and electrolyte content, conductance, and charge density become available will such a discussion and a comparison with physiological membranes become profitable.

#### SUMMARY

1 Experiments were carried out to decide whether or not the electromotive properties of dried collodion membranes depend upon their thickness.

2 A number of dried collodion membranes of varying thickness, 3–160  $\mu$ , were prepared from collodion preparations of different electrochemical activity. The characteristic concentration potentials across them were measured and the means of these values determined for each thickness.

3 The characteristic concentration potentials across dried collodion membranes are a function of their thickness. The thinnest membranes yield in all cases the lowest concentration potentials, increasingly thicker membranes give increasingly higher potential values, until a constant value is reached which is characteristic of the particular collodion preparation used. With electrochemically active collodion, characteristic concentration potentials approaching the thermodynamically possible maximum are obtained with membranes of only 10  $\mu$  thickness, thinner membranes giving appreciably lower values. With two rather inactive commercial collodion preparations the characteristic concentration potential increases from about 30 mv for membranes 3  $\mu$  thick to about 42 mv for 20  $\mu$  membranes; still thicker membranes do not show a significant increase in the potential values. With a highly purified collodion preparation the constant maximum value was found to be about 32 mv, 4  $\mu$  thick membranes giving only about 22 mv.

4 These results do not support the homogeneous phase theory as applied to the dried collodion membrane. They are readily compatible with the micellar-structural theory. Several special possible cases of the latter as applied to the dried collodion membrane are discussed.





entering) at any time is equal to the specific activity of its immediate precursor<sup>3</sup> at that time, and that the specific activity of portions of the compound breaking down (or leaving the tissue) is equal to the specific activity of the total amount of the compound present in that tissue.

### A Criteria for the Establishment of a Precursor

It is known that in a biological system one deals with dynamic equilibrium mixtures of all types of molecules at different energy levels. Isolation of a com

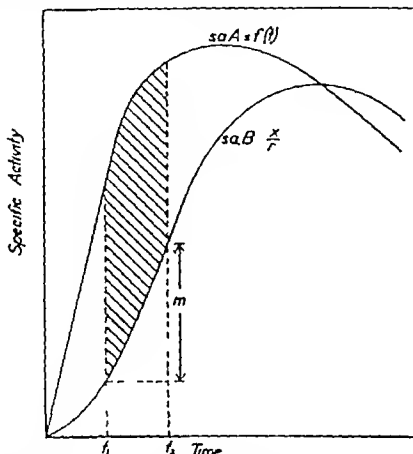


FIG 1 Illustration of the "s.a. time" relations of precursor *A* and product *B*

pound from such mixtures undoubtedly involves the shifting of these equilibria towards that more stable compound. If, therefore, the immediate precursor *A* of a compound *B* can be isolated by chemical procedures, it means that *A* is the last "stable" compound which precedes the formation of *B*. Compound *B* may have more than one immediate precursor, because two or more molecules may combine to form *B*. At present, however we are concerned only with the precursor *A* containing the labeled atom, and we therefore will call it the immediate precursor of *B*.

It has been pointed out frequently that during the early interval after the administration of a labeled substance the s.a. (or isotopic concentration, if non radioactive isotopes are employed) of a precursor of a compound must be

<sup>3</sup> The term immediate precursor is explained below

that the same constant specific activity of the immediate precursor was maintained at the site of the reaction. Whether this is the case is not known. From the ratios of the specific activity of the organ phospholipid to that of plasma phosphate, they obtained the fraction of newly formed phospholipid molecules formed in that organ. They failed to take into account the breakdown of newly formed molecules, hence their calculation is applicable only to experiments of very short duration with respect to the turnover time of a given substance.

In the present communication, a simple method for the determination of the turnover rate of a substance and the identification of its precursor is presented.

### *Terminology and Assumptions*

The following terms are used in the present treatment

*Specific Activity*—The specific activity ( $s_a$ ) of a substance containing a labeled atom,  $L$ , is the amount of radioactive  $L$  (radioactive units) per unit of labeled  $L$  (mg) present.

*Turnover*—This term refers to the process of renewal of a given substance, which may be accomplished in the following ways: (1) The incorporation of labeled atoms or radicals into a substance,  $i.e.$ , synthesis or exchange. (2) The entering of a labeled substance into a tissue,  $i.e.$ , transport. (3) A combination of the above two processes, which may be termed here appearance of a substance.

*Turnover Rate*—The turnover rate of a substance in a tissue is the amount of the substance that is turned over by that tissue per unit of time.

*Turnover Time*—The turnover time of a substance in a tissue is the time required for the appearance or disappearance of an amount of that substance equal to the amount of that substance present in the tissue. If, for example, the rate of appearance of a substance in a tissue is " $a$ " and the amount of that substance present in that tissue is " $b$ ," the turnover time will be " $\frac{b}{a}$ ."

The following assumptions are made in the calculations below

(1) *Steady State*—The amount of compound present in the tissue studied must be constant during the interval over which the calculation is made,  $i.e.$ , the rate of appearance of the compound must equal its rate of disappearance.

(2) *Constant Rate of Appearance and Disappearance*—The rate of appearance and disappearance of the compound must be constant during the time interval used for the calculation.

(3) *Random Appearance and Disappearance*—The appearance and disappearance of all molecules must proceed at random,  $i.e.$ , the organism does not distinguish between "old" and "newly" formed molecules. In the case of phospholipids Hevesy and Hahn (3) appear to assume that such a distinction is made in the animal organism, but this seems very unlikely, especially in tissues where little or no organization exists such as plasma. The assumption made in the present study implies that the specific activity of the compound formed (or

entering) at any time is equal to the specific activity of its immediate precursor<sup>3</sup> at that time, and that the specific activity of portions of the compound breaking down (or leaving the tissue) is equal to the specific activity of the total amount of the compound present in that tissue

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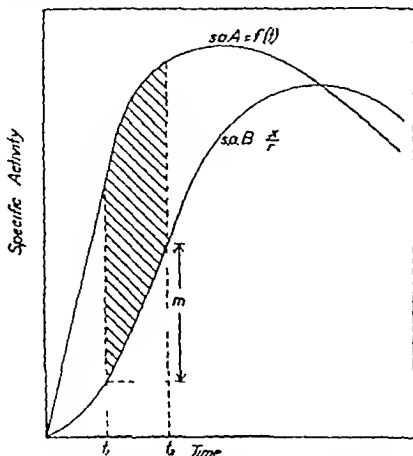


FIG. 1. Illustration of the 's.a.' time relations of precursor *A* and product *B*.

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It has been pointed out frequently that during the early interval after the administration of a labeled substance the s.a. (or isotopic concentration, if non-radioactive isotopes are employed) of a precursor of a compound must be

<sup>3</sup> The term immediate precursor is explained below.

that the same constant specific activity is maintained at the site of the reaction. From the ratios of the specific activity of plasma phosphate, they obtain the number of molecules formed in that organ per unit time. Let us now turn down of newly formed molecules. In experiments of very short duration, the specific activity of the substance remains constant.

In the present communication, we shall discuss the turnover rate of a substance.

The following terms

*Specific Activity*  
The specific activity,  $L$ , is the amount of radioactivity present per unit mass of the substance.

*Turnover* — The turnover time,  $T$ , is the time required for the substance to be completely replaced by new molecules which may be formed in the body.

*Turnover Rate*  
The turnover rate,  $R$ , is the reciprocal of the turnover time,  $T$ .

*Turnover Fraction*  
The turnover fraction,  $f$ , is the fraction of the substance which is replaced per unit time.

is "b,"

T

f

b

c

(1) If the slope of the "s.a. time" curve of  $B$  is positive (see Fig. 1), i.e. before the s.a. of  $B$  reaches its maximum,  $\left[f(t) - \frac{x}{r}\right]$  must be positive. This means that the s.a. of the immediate precursor  $A$  is greater than that of the compound  $B$  before the latter reaches its maximum s.a.

(2) After  $B$  has reached its maximum s.a., the slope of the "s.a. time" curve of  $B$  is negative, and therefore the s.a. of the compound is greater than that of its precursor.

(3) At the time when  $B$  has reached its maximum s.a. the slope of the "s.a. time" curve of  $B$  is zero and therefore the s.a. of the immediate precursor  $A$  equals the s.a. of compound  $B$  at that time.

### B. Calculations of Turnover Time

The general equation derived above was

$$\frac{r}{p} \left( \frac{d\bar{x}}{dt} \right) = \left[ f(t) - \frac{x}{r} \right] \quad (1)$$

As defined above, turnover time (which will be designated by  $t_t$ ) =  $\frac{r}{p}$ . If the "s.a. time" curves of  $A$  and  $B$  are known, it is possible to determine  $\frac{r}{p}$  from equation (1)

Since the determination of the slope  $\frac{d\bar{x}}{dt}$  involves an error much larger than the experimental error in  $\frac{x}{r}$ , it is advisable to use the integrated rather than the differential equation

$$\begin{aligned} \frac{r}{p} \int_{t_1}^{t_2} d\bar{x} &= \int_{t_1}^{t_2} f(t) dt - \int_{t_1}^{t_2} \frac{x}{r} dt \\ t_t \left( \frac{x_2}{r} - \frac{x_1}{r} \right) &= \int_{t_1}^{t_2} f(t) dt - \int_{t_1}^{t_2} \frac{x}{r} dt = \text{shaded area} \\ t_t &= \frac{\text{shaded area}}{m} \quad (\text{see Fig. 1.}) \end{aligned}$$

From Fig. 1 it can be seen how  $t_t$  can be determined. There are cases, however, in which it is advisable to use the ratios of the s.a. of  $B$  to the s.a. of  $A$ , rather than their absolute values. This will be the case, for data from more than one animal are used in the calculation,

higher than that of the compound itself. It has been noted too that, if the *s a* of the precursor is maintained constant, the *s a* of the compound eventually becomes equal to that of the precursor.

It will now be shown how an immediate precursor can be determined more precisely from the "s a -time" relations of the precursor and compound. Let us again consider the case in which a single immediate precursor *A* is converted to compound *B*.

Let

$p$  = the rate of conversion of *A* to *B* (assumed to be constant)

$r$  = the amount of *B* present in the tissue (assumed to be constant)

$x$  = the amount of radioactive *B* present in that tissue

$f(t)$  = the *s a* of the immediate precursor *A*, which, as expressed here, depends on time.

Then the amount of radioactivity that will be converted into *B* per unit of time is  $pf(t)$ , and the amount of radioactivity that is lost from *B* per unit of time is  $p \frac{x}{r}$ . Therefore, the rate of change of the amount of radioactivity in *B* in a tissue per unit of time =

$$\frac{dx}{dt} = pf(t) - p \frac{x}{r} = p \left[ f(t) - \frac{x}{r} \right] \quad \text{or}$$

$$r \left( \frac{d \frac{x}{r}}{dt} \right) = p \left[ f(t) - \frac{x}{r} \right] \quad \text{since } r \text{ is constant}$$

$$\text{and} \quad \frac{\left( \frac{d \frac{x}{r}}{dt} \right)}{f(t) - \frac{x}{r}} = \frac{p}{r} = \text{a constant}$$

$\left( \frac{d \frac{x}{r}}{dt} \right)$  measures the slope of the "s a -time" curve of *B*. We may now deduce the following relation between the *s a* of the compound *B* and the *s a* of its precursor *A*. *At any time the slope of the "s a -time" curve of B is proportional to the difference between the s a of A, i.e.,  $f(t)$ , and the s a of B, i.e.,  $\frac{x}{r}$ .*

The application of this relation in the case in which a single dose of labeling agent is administered is illustrated in Fig. 1. In general the following three criteria for an immediate precursor will be most useful

(1) If the slope of the "s.a.-time" curve of  $B$  is positive (see Fig. 1), *i.e.* before the s.a. of  $B$  reaches its maximum,  $\left[ f(t) - \frac{x}{r} \right]$  must be positive. This means that the s.a. of the immediate precursor  $A$  is greater than that of the compound  $B$  before the latter reaches its maximum s.a.

(2) After  $B$  has reached its maximum s.a., the slope of the "s.a.-time" curve of  $B$  is negative, and therefore the s.a. of the compound is greater than that of its precursor.

(3) At the time when  $B$  has reached its maximum s.a. the slope of the "s.a.-time" curve of  $B$  is zero and therefore the s.a. of the immediate precursor  $A$  equals the s.a. of compound  $B$  at that time.

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As defined above, turnover time (which will be designated by  $t_t$ ) =  $\frac{r}{p}$ . If the "s.a. time" curves of  $A$  and  $B$  are known, it is possible to determine  $\frac{r}{p}$  from equation (1)

Since the determination of the slope  $\frac{d\frac{x}{r}}{dt}$  involves an error much larger than the experimental error in  $\frac{x}{r}$ , it is advisable to use the integrated rather than the differential equation

$$\begin{aligned} \frac{r}{p} \int_{t_1}^{t_2} d\frac{x}{r} &= \int_{t_1}^{t_2} f(t) dt - \int_{t_1}^{t_2} \frac{x}{r} dt \\ t_t \left( \frac{x_2}{r} - \frac{x_1}{r} \right) &= \int_{t_1}^{t_2} f(t) dt - \int_{t_1}^{t_2} \frac{x}{r} dt = \text{shaded area} \\ t_t &= \frac{\text{shaded area}}{\frac{x_2}{r} - \frac{x_1}{r}} \quad (\text{see Fig. 1}) \end{aligned}$$

From Fig. 1, it can be seen how  $t_t$  can be determined. There are cases, however, in which it is advisable to use the ratios of the s.a. of  $B$  to the s.a. of  $A$  rather than their absolute values. This will be the case, for example, when data from more than one animal are used in the calculation, since the above



ratios tend to be more uniform from animal to animal than the  $s a$  themselves. An illustration of the use of these  $s a$  ratios will be given in the following two cases.

The analytical solution of equation (1) is

$$x e^{\frac{p}{r}t} = \int p f(t) e^{\frac{p}{r}t} dt + C$$

For the case where the  $s a$  of the immediate precursor is maintained constant (*i.e.*  $f(t) = a$ ), this becomes  $\frac{x}{r} = a \left(1 - e^{-\frac{p}{r}t}\right)$ , since  $x = 0$  when  $t = 0$ . At turnover time  $t = \frac{r}{p}$  and therefore  $\frac{x}{r} = a(1 - e^{-1}) = 0.63 a$ , *i.e.* the ratio of the  $\frac{s a B}{s a A} = 0.63$  at turnover time, and similarly at  $\frac{1}{n}$  of the turnover time

$$\left(\text{i.e. where } t = \frac{t_t}{n}\right) \frac{s a B}{s a A} = \left(1 - e^{-\frac{1}{n}}\right) \quad (2)$$

When the  $s a$  of the immediate precursor varies linearly with time (*i.e.*  $f(t) = bt$ ), the solution is simplified to

$$\frac{x}{r} = \frac{br}{p} \left(\frac{p}{r}t - 1 + e^{-\frac{p}{r}t}\right) \text{ since } x = 0 \text{ when } t = 0$$

At turnover time again  $t = \frac{r}{p}$  and  $\frac{s a B}{s a A} = \frac{x/r}{bt_t} = 0.37$ ,

$$\text{and at } \frac{1}{n} \text{ turnover time } \frac{s a B}{s a A} = \frac{x/r}{bt_{t/n}} = n \left(\frac{1}{n} - 1 + e^{-\frac{1}{n}}\right) \quad (3)$$

From a given ratio of the  $s a$  of  $B$  to the  $s a$  of  $A$  at the time interval  $T$  we can determine " $n$ " from equation (2) or (3), and from  $T = \frac{t_t}{n}$  the turnover time of the given substance  $B$  can be determined.

If the total amount of substance  $B$  present in that tissue is known ( $= r$ ), the turnover rate " $p$ " can be obtained from the equation  $\frac{r}{t_t} = p$ .

*The Rate of Disappearance of a Compound as a Measure of Turnover Rate*—If the turnover rate of a substance  $B$  in the circulating fluid has to be determined, it is convenient to measure its rate of disappearance from the circulating fluid. From the "steady state" assumption we know that the rate of appearance of  $B$  in the circulating fluid must equal its rate of disappearance, so that the latter gives a true measure of the turnover rate. The advantage of this method is that the immediate precursor of the compound  $B$  does not have to be known.

The measurement can be performed by the introduction of a small amount of

labeled substance  $B$  into the circulating fluid and by determining its s.a. at different time intervals thereafter

Let

$p$  = rate of disappearance of  $B$  from the circulating fluid,

$x$  = the amount of radioactive  $B$  (in r u.) present in the circulating fluid at any time,

$r$  = the total amount of  $B$  present in the circulating fluid (assumed to be constant)

then

$$\frac{dx}{dt} = -p \frac{x}{r} \text{ and on integration } \frac{x}{r} = ce^{-\frac{p}{r}t},$$

taking the natural logarithm on both sides,  $\ln \frac{x}{r} = \ln c - \frac{p}{r}t$ . It is clear

that a plot of  $\ln \frac{x}{r}$  ( $= \ln \text{s.a. } B$ ) against  $t$  will yield a straight line whose slope will be  $= -\frac{p}{r} = -\frac{1}{t_i}$  (4)

From equation (4) the turnover rate  $p$  can also be determined if the total amount of  $B$  present in the circulating fluid ( $= r$ ) is known. The latter quantity can be calculated from the data obtained in this type of experiment, as will be shown in the following paper dealing with the determination of the turnover of phospholipids in the plasma of dogs. It should be kept in mind that the above relations will hold only during a time interval in which no appreciable amount of isotopic substance returns from the tissues to the circulating fluid

The suggestions and assistance of Professor L. L. Chaikoff in the preparation of this manuscript is gratefully acknowledged

#### SUMMARY

1 A new method for the determination of an immediate precursor of a substance occurring in the animal body is presented.

2 Calculations on the quantitative determination of the rate of turnover of a substance and their application to experiments involving the use of labeling agents are given. These calculations take into account loss of the isotopic substance by way of breakdown or transport

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# THE TURNOVER RATE OF PHOSPHOLIPIDS IN THE PLASMA OF THE DOG AS MEASURED WITH RADIOACTIVE PHOSPHORUS

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From the considerations presented in the previous paper (1), it is apparent that an exact measure of the turnover of a compound in an organ like kidney liver, etc. requires knowledge of the "specific activity time" relations of (1) the compound itself and (2) its precursor. For the determination of the turnover rate of phospholipid phosphorus of plasma, however, the procedure can be greatly simplified, since it is feasible to introduce a small amount of plasma containing the labeled compound and measure its disappearance from the circulating fluid. This procedure makes unnecessary the measurement of the specific activity of the immediate precursor, and so the path of formation of the compound need not be known. The compound, in its labeled form, can be either obtained by synthesis *in vitro* or used as present in plasma removed from an animal that has synthesized the labeled compound. The latter procedure has the advantage in that the labeled phospholipid introduced is of the same type as that present in plasma and its physicochemical state is not altered by chemical treatment. In other words, the isotopic phospholipid whose disappearance is being measured is chemically indistinguishable from the animal's own plasma phospholipid. The above principles have been applied in the present investigation to determine the rate at which phospholipid phosphorus is turned over in the plasma of the dog.

## EXPERIMENTAL

Dogs 1A and 2A served as donors. They received intraperitoneal injections of 2 to 3 millicuries of labeled inorganic phosphate. 26 hours later, blood was removed from the dogs, heparinized, and plasma separated by centrifugation.

The turnover of plasma phospholipid was determined in dogs 1B, 2B, and 3B. The following amounts of blood were removed from these dogs: 50 cc. from dog 1B, 20 cc. from dog 2B, and 20 cc. from dog 3B. Immediately thereafter 100 cc. of radioactive plasma obtained from dog 1A was injected into the femoral vein of dog 1B, whereas dogs 2B and 3B were injected with 70 cc. of radioactive plasma obtained from dog 2A. During the next 5 hours eight blood samples were removed from dogs 1B, 2B, and 3B for the determination of total phospholipid, total  $P^{32}$ , and phospholipid  $P^{32}$ . During this time, a total of 50 cc. of blood was removed from dogs 1B, 2B, and 3B. After removal of the last blood sample, the three dogs were sacrificed by means of an intravenous injection of nembutal. Liver, kidney, small intestine, muscle, and spleen



as follows: 1 cc. of plasma was transferred dropwise to a centrifuge tube containing 10 cc. of 10 per cent trichloroacetic acid and the mixture vigorously agitated with a glass rod. It was allowed to stand for 10 minutes, during which it was thoroughly agitated several times to bring the precipitate to a finely suspended state. The mixture was then centrifuged for 5 minutes, the supernatant fluid decanted, the precipitate washed once with 5 cc. of 10 per cent trichloroacetic acid, and the suspension again centrifuged. The precipitate was dissolved in a few drops of 10 per cent NaOH and quantitatively transferred to a volumetric flask, aliquots were then taken for the determination of  $P^{32}$ .

The radioactive units expressed as counts per minute for (1)  $P^{32}$  content of the trichloroacetic acid insoluble fraction, (2) the phospholipid  $P^{32}$ , and (3) total  $P^{32}$  for plasma of donor and recipient are recorded in Table I.

Table I shows a striking agreement between the values for the  $P^{32}$  content of the trichloroacetic acid precipitate obtained from plasma and the  $P^{32}$  content of the petroleum ether extract prepared from plasma. In order to avoid the removal of excessive amounts of blood from dogs 1B, 2B, and 3B, the  $P^{32}$  content of the trichloroacetic acid precipitate of plasma was taken as equivalent to its phospholipid  $P^{32}$  content.

### RESULTS

In the preceding paper the following equation was derived for the disappearance of phospholipid phosphorus from the circulating fluid

$$\frac{x}{r} = ce^{-\frac{p}{r}t} \quad (1)$$

or

$$\frac{x}{f} = \left(\frac{r}{f}\right) ce^{-\frac{p}{r}t} \quad (2)$$

in which

- $p$  = rate of disappearance of phospholipid phosphorus from the circulating fluid (this is assumed to be constant)
- $x$  = the amount of phospholipid  $P^{32}$  (counts per minute) present in the circulating fluid at any time,
- $r$  = the total amount of phospholipid phosphorus (mg.) present in the circulating fluid (this is also assumed to be constant)
- $f$  = the amount (cc.) of circulating fluid in the organism.

Therefore,  $\frac{x}{f}$  gives the counts of phospholipid  $P^{32}$  per cc. of plasma at any time and  $\frac{r}{p}$  is equal to the turnover time of phospholipid phosphorus in the

circulating fluid, while  $\frac{r}{f}$  is a measure of the phospholipid phosphorus level  $\left(\frac{\text{mg}}{\text{cc}}\right)$  in the circulating fluid  
 Since

$$\log \frac{x}{f} = \log \left( c \frac{r}{f} \right) - \frac{p}{r} t \log e, \quad (3)$$

the slope of the straight line (Fig 1) obtained by plotting the  $\log$  of the  $P^{32}$  values for the trichloroacetic acid precipitate per cc of plasma  $\left( c e \log \frac{x}{f} \right)$  against time ( $t$  = minutes after the injection of plasma) is  $\frac{p}{r} \log e$

TABLE II  
 Values for  $f$ ,  $t_i$  and  $p$  in Plasma

Dog		1B	2B	3B
Plasma volume ( $f$ )	Cc	535	323	339
	Per cent of body weight	6.6	4.9	6.0
Turnover time ( $t_i$ ), hrs		9.3	7.2	7.8
Turnover rate ( $p$ ), mg phospholipid phosphorus		7.0	5.2	8.0

*Turnover Time*—Turnover time for phospholipid phosphorus can be determined from Fig 1. Thus

$$t_i = \frac{r}{p} \text{ and from equation (3) the slope of the curve is } -\frac{p}{r} \log e, \text{ therefore}$$

$$t_i = -\frac{\log e}{\text{Slope}} = -\frac{0.434}{\text{Slope}}$$

The values for turnover time for phospholipid phosphorus of dogs 1B, 2B, and 3B are shown in Table II

*Turnover Rate*—To determine the turnover rate of phospholipid phosphorus in the circulating fluid it is necessary to know  $t_i$  and the total amount of phospholipid phosphorus present in the circulating fluid. The latter can be obtained from two measurements, (1) the concentration of the phospholipid phosphorus in the circulating fluid (mg per cc), and (2) the volume of the circulating fluid of the dog. The amount of the circulating fluid for each dog can be determined as follows

The slopes of the curves in Fig 1 are constant after the 20 minute interval

and smaller than the slopes between 0 and 20 minutes. The greater slopes found during the early intervals may be explained by supposing a non-uniform distribution of the injected radiophospholipid in the circulating fluid during

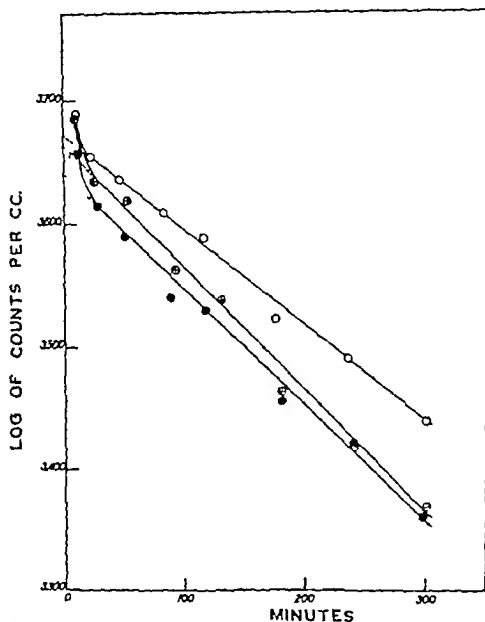


FIG. 1 The logarithm of the counts per cc. of plasma phospholipid phosphorus as plotted against time.  $\circ$  = Dog 1B  $\odot$  = Dog 2B  $\bullet$  = Dog 3B

this interval. There is no reason to doubt that a uniform distribution of the injected phospholipid phosphorus had occurred at the later intervals. For this reason one is justified in extrapolating the straight line curves to "zero-time" in order to obtain the number of counts per cc. of phospholipid  $P^{32}$  that would have been present in the circulating fluid if the specific activity of the phospholipid phosphorus through all parts of the circulating fluid had been the same at "zero-time." Mathematically, this corresponds to determining



$\log \left( c \frac{r}{f} \right)$  When  $t = 0$ ,  $\log \left( c \frac{r}{f} \right) = \log \frac{\lambda}{f}$  Therefore by dividing the number of counts of phospholipid  $P^{32}$  per cc of plasma at "zero-time"  $\left[ \left( \frac{x}{f} \right)_{t=0} \right]$  into the total amount of phospholipid  $P^{32}$  injected, the volume in which the phospholipid  $P^{32}$  has distributed itself is obtained

The values for the volume of circulating fluid ( $f$ ) are shown in Table II. These values agree with plasma values obtained in dogs by the dye-injection methods (4). So it is reasonable to assume that the injected phospholipid is *initially mixed only with plasma* of the recipient dogs and that the penetration of injected phospholipid into other tissues is a relatively slow process.

The turnover rate of phospholipid phosphorus ( $p$ ) can now be obtained from the total amount of phospholipid phosphorus in the plasma ( $r$ ) and its turnover time ( $t_t$ ) as follows

$$p = \frac{r}{t_t}$$

The values for  $p$  are recorded in Table II.

#### DISCUSSION

Previous studies of the fate of injected labeled phospholipid were made by Hahn and Hevesy (5) and by Haven and Bale (6). The latter injected emulsions of labeled phospholipid extracted from rat tissues and determined its disposition in the tissues of the rat. Hahn and Hevesy, however, injected plasma containing labeled phospholipid. Although compounds other than phospholipid are injected into the animal when plasma is employed, it has been shown in this laboratory that these cause no appreciable increase in radioactive phospholipid synthesis in the liver in the interval studied. Thus 2,540,000 radioactive units were injected into dog 1B, of this amount 600,000 were present in compounds other than phospholipid. It has been shown that in 5 hours 0.01 per cent of injected inorganic  $P^{32}$  is converted to phospholipid per gm of liver in the dog (7). Of the 14,400 counts of phospholipid  $P^{32}$  found per gm of liver in dog 1B, 60 counts were probably synthesized from phosphate injected in forms other than phospholipid.

The distribution of phospholipid  $P^{32}$  in the tissues of the three dogs 5 hours after the injection of the labeled plasma is shown in Table III. A large fraction (51–58 per cent of that injected) of the phospholipid  $P^{32}$  is still present

<sup>1</sup> These values are sufficient to give the turnover time of plasma phospholipid phosphorus once it has been established that the curves in Fig. 1 are straight lines. Thus by substitution in equation (1)

$$0.58 = 1 e^{-5/t_t}, \quad t_t = 9.1 \text{ for dog 1B,}$$

$$0.51 = 1 e^{-5/t_t}, \quad t_t = 7.5 \text{ for dog 2B,}$$

$$0.53 = 1 e^{-5/t_t}, \quad t_t = 7.8 \text{ for dog 3B,}$$

in the plasma at the end of 5 hours. At complete turnover time (i.e. after 9 hours for dog 1B, after 7 hours for dog 2B, and after 8 hours for dog 3B) 37 per cent of the injected phospholipid  $P^{32}$  would still be present in the plasma

TABLE III

*Distribution of Phospholipid (PL) in Tissues at the End of 5 Hours*

1	Dog 1B			Dog 2B			Dog 3B		
	2	3	4	5	6	7	8	9	10
	Per cent of injected dose PLPm per whole organ after 5 hrs.	Amount of PLP per whole organ	Per cent of Organ's PL supplied by plasma PL per hr.†	Per cent of injected dose PLPm per whole organ after 5 hrs.	Amount of PLP per whole organ	Per cent of Organ's PL supplied by plasma PL per hr.†	Per cent of injected dose PLPm per whole organ after 5 hrs.	Amount of PLP per whole organ	Per cent of Organ's PL supplied by plasma PL per hr.†
		mg			mg			mg	
Plasma	57.7	12.2§	—	51.4	11.6§	—	53.0	18.5§	—
Liver	16.2	255	1.08	15.2	169	0.93	11.1	173	1.09
Kidney	0.76	31.6	0.41	1.27	24	0.35	1.13	28.6	0.67
Small intestine	—	—	—	2.59	54	0.50	2.44	68	0.61
Spleen	—	—	—	0.30	8.2	0.38	0.35	8.5	0.71
Cells	0.36	—	—	0.35	—	—	1.15	—	—
Muscle	7.4**	—	—	4.7**	—	—	4.2**	—	—

\* PLP refers to phospholipid phosphorus

† Breakdown of phospholipid  $P^{32}$  is disregarded.

§ Mg phospholipid phosphorus per 100 cc.

|| Washed twice with Ringer's solution.

\*\* Analysis of gastrocnemii muscles was made. Values for whole organ based on muscle constituting 40 per cent of body weight.

This can be shown as follows. By substitution of  $t = \frac{r}{p}$  in equation (1),

$$\frac{x}{r} = ce^{-1} = 0.37c$$

where  $c$  = the specific activity of the plasma phospholipid phosphorus at "zero-time"

Next to plasma, the liver contained the largest amounts of phospholipid  $P^{32}$ . These findings are in agreement with those reported by Hahn and Hevesy (5), and by Haven and Bale (6).

Information about the transport of plasma phospholipid to the different organs can be obtained from the data presented here. In columns 4, 7, and 10 of Table III is shown the percentage of each organ's phospholipid that is

supplied per hour by the plasma<sup>2</sup> In the case of the liver, 1 per cent of its phospholipid is obtained directly from plasma phospholipid per hour In the kidney and small intestine, about 0.5 per cent is so derived In these calculations we have chosen to neglect loss of the phospholipid  $P^{32}$  in the organs examined since no reliable data on breakdown or leaving of phospholipid are available The data in Table III (columns 2, 5, and 8) show that as much as 76–83 per cent of the injected phospholipid  $P^{32}$  can be accounted for by the seven tissues examined in the present investigation These values would appear to exclude a rapid breakdown of phospholipid in the animal

#### SUMMARY

1 A method for the determination of turnover time and turnover rate of plasma phospholipid is presented

2 During the postabsorptive state 5.2 to 8.0 mg of phospholipid phosphorus are turned over per hour in the plasma of dogs weighing 6–9 kilos

3 The amount of phospholipid in an organ that is supplied by plasma phospholipid per hour is calculated

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<sup>2</sup> Let "q" be the rate of transport of plasma phospholipid to an organ and "a" be the number of counts of phospholipid  $P^{32}$  present in an organ at time  $t$ . Since the specific activity of plasma phospholipid phosphorus equals

$$\frac{x}{r} = ce^{-\frac{p}{r}t}, \quad a = \int_0^t qce^{-\frac{p}{r}t} dt$$

by integration

$$q = \frac{a}{c \left[ \frac{e^{-\frac{p}{r}t} - 1}{-\frac{p}{r}} \right]} = \frac{a}{c \left[ 1 - e^{-\frac{p}{r}t} \right] t}$$

For the liver of dog 1B

$$q = \frac{412,000}{\frac{4710}{0.122} \left[ 1 - e^{-\frac{6}{9.34}} \right] 9.34} = 2.76$$

i.e., 2.76 mg of phospholipid phosphorus enters the liver of dog 1B per hour. Hence the percentage of the liver phospholipid supplied by plasma phospholipid =  $\frac{2.76 \times 100}{255} = 1.08$  per cent. Hevesy and Hahn (8) used a similar approach to calculate the percentage of plasma phospholipid transferred from plasma to liver

# SEDIMENTATION IN THE ANGLE CENTRIFUGE

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PLATES 1 TO 3

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Differential centrifugation in a high speed angle centrifuge has become a standard procedure for the purification and concentration of viruses and other biological materials of submicroscopic size. The degree of centrifugation necessary for a given material has generally been determined by trial, since experimental data have not been available which would allow a prediction, with any certainty, as to just how particles of known size would behave in an angle centrifuge. Experimental conditions in the angle centrifuge are quite different from those afforded by a sector-shaped ultracentrifuge cell of the Svedberg type (1), in which the side walls are directed toward the axis of rotation, so that a particle originally near any one of them continues its own radial migration parallel to the surface and without interference with its movement. If a general correlation could be established between sedimentation in the angle centrifuge and in the ultracentrifuge, which exhibits a discrete and measurable sedimentation boundary indicative of the particle size, it would then be possible not only to predict the behavior of a known material in the angle centrifuge but to estimate an unknown particle size from experimental data.

For reasons already cited, it has been impossible to interpret satisfactorily certain results obtained with angle centrifuges during the course of investigating several animal viruses in this laboratory. Among the primary observations have been the following: the presence of residual infectivity in the supernatant fluid of virus suspensions even after prolonged centrifugation (2), the variation in the degree of sedimentation obtained under identical experimental conditions with different concentrations of the same material (3), the different degrees of sedimentation accomplished with equal concentrations of the same material centrifuged under conditions which differed only in that one rotor was spun in a vacuum and the other in the open air. Among other puzzling observations which have awaited satisfactory explanation has been that of Claude (4), who reported the appearance of abnormally sharp boundaries of *Limulus* hemocyanin in a high speed electrically driven angle centrifuge spinning in the open air. It would be expected that the normal diffusion of particles during the long centrifugation period required would have caused pronounced blurring of the boundary.

With the view of clarifying conceptions of the process by which sedimenta

tion is accomplished in angle centrifuges, a systematic investigation of the problem was undertaken with rotors spinning both in the open air (5) and in a vacuum (2). In order to simulate conditions encountered with biological agents of especial interest, *i.e.*, the smaller viruses, it was considered advantageous to use some readily accessible, homogeneous material having a comparable particle size.

### *Material and Methods*

The material selected for use in all experiments was the hemocyanin from *Limulus polyphemus*. This large respiratory protein has a molecular weight of several millions (6) and, as has been observed during experiments to be described in a subsequent report, almost all of the protein exists in the form of a component having a sedimentation constant of about  $57 \times 10^{-13}$  cm/sec/unit field in an appropriate buffer solution of pH 6.9 or in the native serum when properly handled. For most of the work, the protein was first purified by several high and low speed centrifugations and then suspended in a buffer solution containing 1 per cent NaCl. In other cases, the native serum was only centrifuged several times at low speed to remove the jelly-like material and other coarse particles. It was then diluted to the required protein concentration by the addition of the buffer solution. Concentration, sedimentation rate, and the certainty of homogeneity were determined by analysis with a refractive index method (7) in the vacuum type ultracentrifuge of Bauer and Pickels (8). Experiments were performed with solutions having hemocyanin concentration ranging from 0.04 to 1.6 per cent.

Similar runs were made with two high speed angle centrifuges, one electrically driven and spinning in the open air (5) and the other driven by compressed air (2) and spinning in high vacuum. In both instances use was made of 20 cm duralumin rotors which accommodated transparent celluloid tubes 9 cm in length and 1.3 cm in diameter (2). The angles of the tubes for the electrical and vacuum centrifuges were 40° and 35°, respectively.

In order that a direct comparison might be made between the behavior of the protein in the ultracentrifuge and in the angle centrifuges, the level of the solution in the celluloid tubes and in the ultracentrifuge cell was so adjusted that the meniscus during centrifugation would in every case be exactly the same distance from the axis of rotation, namely, 5.9 cm (Text-fig. 2). Comparison runs were always performed at the same speed, namely, 16,200 R.P.M., and for the same "equivalent sedimentation time." Determination of the equivalent time involved allowances for the different effective times of acceleration and deceleration, corrections to compensate for the lowered viscosity of the solution in the slightly warmer electrical centrifuge, and adjustments for the slower sedimentation in the more concentrated preparations. With certain special exceptions, routine procedures were followed with the electrical and vacuum centrifuges of using normal acceleration times of about 3 minutes and 9 minutes, respectively, and deceleration times of 7 minutes and 9 minutes, respectively, with deceleration especially gradual just before stopping the centrifuge. Just after each tube was filled initially to the required level, a column of clear paraffin oil was carefully added above the aqueous surface to give the meniscus better definition, to prevent evaporation, and for other reasons explained below.

In the higher concentrations of hemocyanin, its blue color permitted the displacement of a sharp boundary in a transparent celluloid tube to be detected and measured visually after the tube had been carefully removed from the rotor and slowly oriented to an upright position. In order to study more exactly the distribution of concentration, particularly in the dilute solutions all tubes were photographed on a specially fitted optical bench. To avoid severe refraction of light by the curved surface of the tube, it was almost completely immersed in water within a small chamber having two flat, parallel glass sides at right angles to the optical path. Light was permitted to pass through only a 0.5 cm vertical section of the tube. A large condensing lens and a ground glass screen were employed to provide an even diffuse illumination over the full length of the tube. From a mercury arc, monochromatic ultraviolet light of 3650 Å, which is strongly absorbed by hemocyanin was isolated by a filter of nickel oxide. A reprojector lens having a focal length of 50 cm was used to minimize the errors of parallax.

The magnification in every case was adjusted to such a value that a direct comparison in terms of radial distance could be made between the photograph of the tube and an absorption photograph of the same material taken in the ultracentrifuge after an equivalent time of sedimentation. In other words when the photographs were placed side by side with the menisci matching any other two matching levels represented equal distances from the axis of rotation, considering the photograph of the tube to characterize conditions which existed along its axis just before the end of the run. This was nearly true since the horizontal angle (with respect to axis of rotation) subtended by the tube was small, and although the meniscus or the thin layer of solution representing a sharp boundary was vertical and slightly curved in the centrifugal field its intersection with the axis of the tube changed only slightly when it was reoriented to a horizontal position in the gravitational field. As illustrated in photographs presented in Fig. 1 the meniscus frequently failed, because of surface tension to reorient itself into a horizontal position. Care was taken on this account to photograph tubes in a direction which was tangential with respect to their position in the centrifuge.

Several different photographic exposures were made for each tube. It was determined experimentally what exposures were necessary to produce equal intensities with a clear fluid when the Svedberg absorption method (1) was used in conjunction with the ultracentrifuge. Since a cell of 1 cm thickness was employed the total absorption by a given solution was roughly the same as when light was directed through the same material held in a tube. Light passing through the clear oil layer above the solution furnished a convenient reference for judging the presence of light absorbing material in the supernatant fluid.

The standard columns of solution in both the electrical and vacuum angle centrifuges had lengths corresponding to a radial distance of about 3.4 cm. In addition, studies were made with shorter columns having a projected radial length of 1.35 cm., which corresponded to the standard column length employed in the analytical ultracentrifuge. These shorter columns (viz. *d* Fig. 1) with menisci at the standard radial distance were prepared by first placing in the tube a proper amount of a heavy non miscible fluid namely, bromobenzene. Also since theoretical consideration has suggested the importance of density gradients within the solution in inhibiting convective disturbances experiments were performed with long columns of solution in which

synthetic density gradients had been provided. The gradient was produced by first preparing two hemocyanin solutions of exactly the same composition (in terms of grams per liter of final solution) except that 8 to 10 per cent sucrose was incorporated in one. Enough of the latter was placed in the tube to form about half the required column. Above this was gently added the second solution to the correct level. A few vertical strokes with a glass rod partially mixed the two fluids, giving a rather uneven but usable gradient. In figuring the equivalent sedimentation time, allowance was made for the increased viscosity of the section through which the boundary migrated. The correction amounted to only a few per cent in all cases.

In order to conduct experiments in which the effects of the deceleration forces and of any possible thermally activated convection could be differentiated from those connected with the sedimentation process, it was necessary to prepare artificial sedimentation boundaries of hemocyanin (*viz a*, Fig 1). These were made with a clear buffer solution and a solution of hemocyanin against which it had been dialyzed to establish an equilibrium of the salts. The clear solution was placed in a celluloid tube, and the heavier solution was then slowly added through a long hollow needle extending to the bottom of the tube and connected to a syringe exhausted gradually by an electrically driven mechanical system (9). Since in most of the sedimentation work a centrifugation time equivalent to 135 minutes at 16,200 R.P.M. and room temperature was employed, the artificial boundaries were made up at distances from the meniscus corresponding to the radial level reached by the boundary in the ultracentrifuge after this time, namely, 6.9 cm (or 1.0 cm from the meniscus, *viz l*, Fig 1).

#### EXPERIMENTAL

It was found possible to make reasonably sharp artificial sedimentation boundaries by the method cited, especially with the higher concentrations of protein. It was shown experimentally that tubes containing the artificial boundaries could be subjected without appreciable effect to all the disturbing influences (except rotation of the centrifuge) encountered in handling those containing real, sedimented boundaries. Blurring of a boundary after the tube had stood for 135 minutes within a stationary rotor and had been subjected to two reorientations between the vertical and inclined positions was only slightly more than that to be expected by reason of the normal diffusion process (*viz a*, Fig 1).

To test for other disturbances not related to sedimentation, artificial boundaries which had stood for 135 minutes were run to the same speed of 16,200 R.P.M. used for the sedimentation experiments, they were then immediately decelerated according to normal procedure. Sedimentation under these conditions could not have been appreciable. Some of the results are illustrated in Fig 1. With 0.8 per cent solutions, no alteration of the boundary was noted in either long or short columns run in the vacuum centrifuge (*viz f*). In the electrical the boundary remained sharp and was displaced away from the meniscus in short columns (*viz d*). In long columns, it was displaced with considerable blurring toward the meniscus (*viz e*). When concentrations of

0.2 per cent were used, mixing was complete with both long and short tubes in the electrical centrifuge (*vis b*), while in the vacuum type the boundary was not completely eradicated but suffered a great deal of spreading, even into the region of the meniscus (*vis c*). Taking the electrical centrifuge to and from a speed of only 1000 R.P.M. caused only partial mixing, roughly comparable to that in tube *c*, Fig 1.

With one exception, runs in both angle centrifuges at 16 200 R.P.M. for an equivalent time of 135 minutes showed no detectable boundaries with a concentration of 0.04 per cent although a faint one was visible in photographs taken with the ultracentrifuge. When a concentration gradient of sucrose was employed in connection with the vacuum centrifuge, a small gradation in the concentration of protein could be vaguely discerned. With the concentration increased to 0.12 per cent in the same centrifuge, better clearing (*vis s*, Fig 2) of the solution was obtained, without boundary formation. Boundaries were barely discernible with contents of 0.2 per cent and still widely spread when the value was increased to 0.36 per cent (*vis m*, Fig 1). At 0.8 per cent, fairly well defined boundaries were had. With the latter concentration boundaries in short columns of fluid were found to be displaced a shorter distance from the meniscus than were those in long columns. The same was true of boundaries formed in the same material when run in the electrical centrifuge (*vis o* and *p*, Fig 1). Using a very gradual deceleration (2 hours) improved the definition of weak boundaries slightly. As illustrated by *n*, Fig 1, and *t*, Fig 2, a considerable improvement was given by the use of a synthetic density gradient.

In the electrical centrifuge, there was no trace of a boundary and no gradation of concentration in long columns with protein concentrations of 0.12, 0.2 and 0.36 per cent (*vis j*, Fig 1 and *v*, Fig 2). There was little, if any, separation of protein from the body of the fluid. With the highest of these concentrations contained in short columns, some clearing of the fluid was noted. The addition of a sucrose gradient to long columns identical to those above made possible the attainment of measurable boundaries in all cases (*vis k*, Fig 1, and *w*, Fig 2). Without the synthetic gradient, concentrations of the order of 0.8 per cent were required to obtain boundaries (*vis g* and *p*, Fig 1). Even when the centrifugation time was doubled and the boundary in such a preparation allowed to approach the bottom of the tube, it continued to remain abnormally sharp (*vis g*). However the concentration of protein in the supernatant fluid increased (compare *p*). When sucrose gradients were added to long columns of 1.08 per cent protein the amount of hemocyanin residing in the supernatant fluid decreased considerably (*vis h* and *i*).

In general with the electrical centrifuge, boundaries without sucrose were either very sharp or non-existent, depending on the concentration of protein. There was a uniform distribution of protein below the boundary, as well as in the supernatant, where the concentration increased with the centrifugation

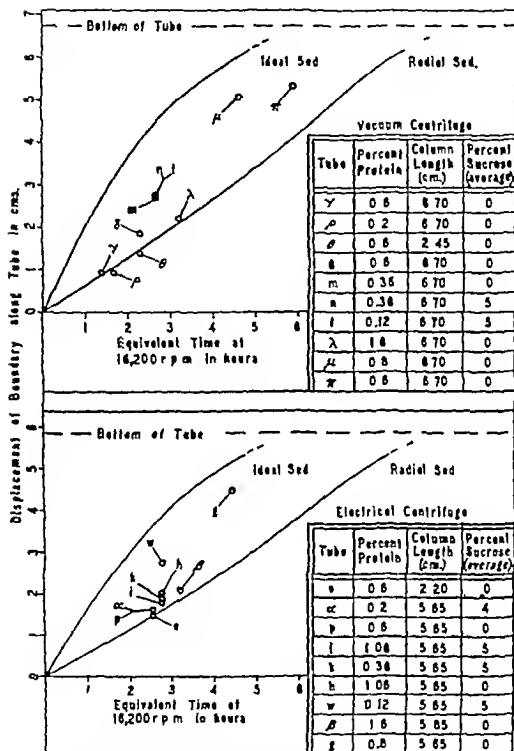


time Obviously, some process was continually active throughout centrifugation maintaining the sharpness of the boundary In the vacuum centrifuge, boundaries became better defined with increasing concentration, and simultaneously a pronounced gradient in concentration from the boundary to the bottom of the tube became evident Supernatant fluids were practically free of protein

In view of the experimental evidence cited, the absence of definition with boundaries of low concentration in the vacuum centrifuge could be explained by disturbances encountered during deceleration and were not necessarily connected with the sedimentation process With both centrifuges, sucrose gradients caused improvement in every instance Low concentrations in the vacuum centrifuge were made to behave more nearly like higher concentrations without sucrose In the electrical centrifuge, the results of the sedimentation process were altered radically and made to resemble more closely those obtained with the vacuum centrifuge Only a small proportion of the effect could have been accounted for by the slightly increased viscosity of the graded sucrose solutions

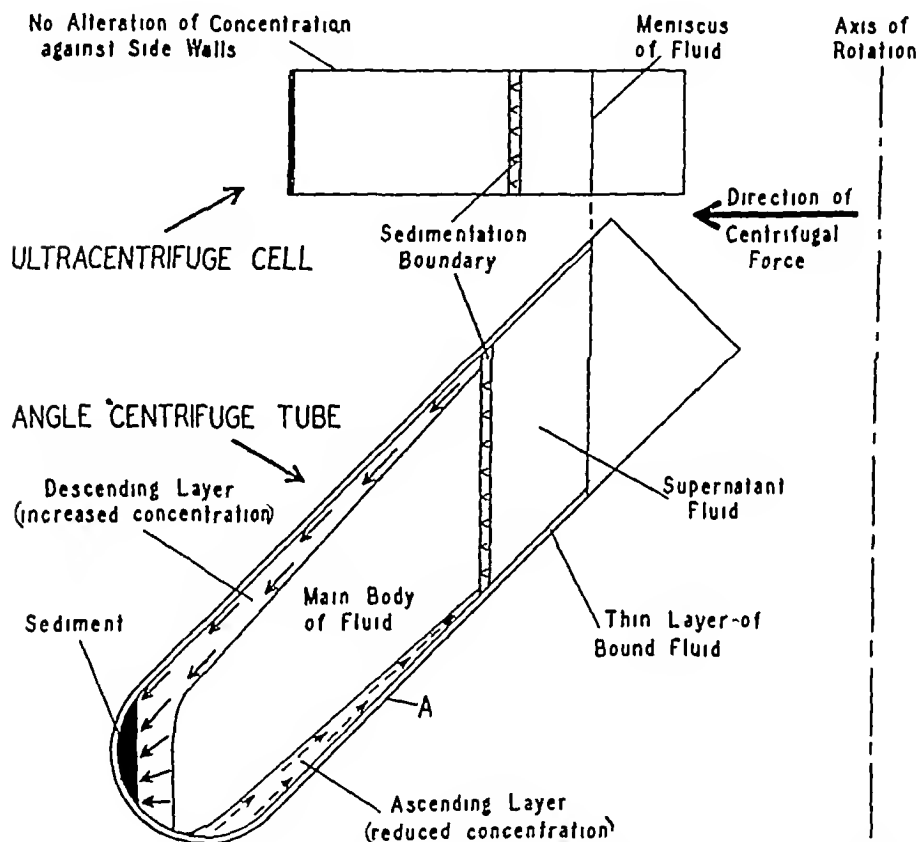
As shown in Text-fig 1, all boundary displacements which could be measured were plotted against the equivalent time of centrifugation Because of the low light absorption and for other reasons already cited, values for the lower concentrations can be regarded only as very rough approximations Within experimental error, the plotted displacements are equal to or greater than those which would have been obtained if the boundaries had progressed away from the axis of rotation at the same rate, measured radially (*radial curve*), as they would have in the analytical ultracentrifuge The displacements were less than if the boundaries had progressed in a certain ideal manner (*ideal curve*) which will be discussed below

Some experiments were also carried out in the ultracentrifuge with the cell turned, in a plane perpendicular to the axis of rotation, about  $65^\circ$  from its normal position In a rough way this arrangement simulated conditions existing in an angle centrifuge Comparative runs were made with the cell oriented normally, the amount of fluid being so adjusted that the meniscus was situated at the same radial level Typical results are illustrated by the refractive index photographs of Fig 3 and the absorption pictures of Fig 2, which show, respectively, the distribution of concentration gradient and of the concentration itself in paired experiments made under identical conditions The serial absorption photographs *q* and *r* (Fig 2) were taken of 0.36 per cent hemocyanin solution at corresponding centrifugation times Those taken with the tilted cell (*r*) show abnormal and progressive alterations of the concentration, being in the direction of decrease just below the boundary and increase in the lower quarter of the column Refractive index photographs *C* and *D* (Fig 3) correspond to the fourth pictures in sets *q* and *r* (Fig 2), respectively



TEXT FIG. 1. Experimental results obtained with various concentrations of hemocyanin compared with results which would be obtained if sedimentation took place as in an ultracentrifuge (radial sedimentation curve) or, as would be expected in an ideal case (ideal sedimentation curve) for which sedimentation near the tube wall is fully effective. Synthetic density gradients made with sucrose were used where its presence is indicated.

As the photographs show, the boundary in the misaligned cell was fairly well defined and was displaced only very slightly more in a given time than was the normal boundary. The abnormal concentration gradient introduced between the boundary and the bottom of the cell is clearly evident in *D*. Photographs *A* and *B* are analogous to *C* and *D*, respectively, except that a protein concentration of only 0.04 per cent was employed for the runs. The



TEXT-FIG 2 Schematic illustration of the sedimentation process in an angle centrifuge as contrasted with that in an ultracentrifuge cell

behavior of the material in this case was of the same general nature except that the displacement of the boundary in the misaligned cell was significantly greater than in the one correctly oriented. The effect was considerably more pronounced than with higher concentrations and was proportionately greater during the early stages of sedimentation.

*Theory of Sedimentation in the Angle Centrifuge*—A general theory which explains the experimentally observed behavior of sedimenting material in the angle centrifuge can be best discussed by reference to Text-fig 2. Consider first a suspension of an ideal material, one which is homogeneous and non-

diffusible, sedimenting in a centrifuge which is entirely free from convective disturbances. Initially, every particle suffers a displacement proportional to and in direction of the applied centrifugal field at the respective starting point. The particle would continue to move in this direction alone if it were not influenced by other factors. Those particles originally at the meniscus do continue the radial movement and form a sedimenting boundary. The initial radial movement of the particles near the walls causes a clearing of the fluid along the inner surface and a deposition of particles against the outer wall. Because of increasing centrifugal force with radial distance, the thickness of the initial cleared layer and the amount of deposition will be greatest in the bottom sections of the tube. Within the body of the uncleared fluid itself, the divergent movement of the particles causes the concentration to decrease slightly with time, but at a rate which is uniform throughout so that homogeneity continues to exist in this region.

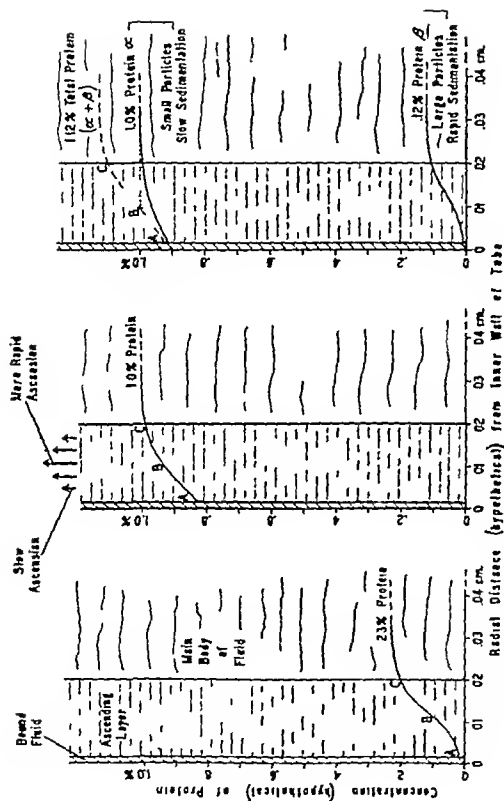
Such a system will continually attempt to adjust itself to the most stable state possible. Any element of fluid which has its density changed from that of its surroundings will seek a new radial level where the density is equal to its own. In accordance with this principle, there is established along the inner wall an ascending layer of clear fluid which rises just above the boundary into the supernatant fluid. The volume of the supernatant fluid is thus increased and the boundary displaced more rapidly than the particles within the boundary actually sediment through the fluid. By a process of integration applied along the inner surface of a given tube for all positions of the boundary, theoretical curves for an ideal material can be constructed which show the progression of a boundary as related to the normal sedimentation of the material in an ultracentrifuge. Curves of this type, based on a normal sedimentation rate equal to that of *Limulus* hemocyanin, have been determined for the full length columns of fluid used in the present studies. They are represented in Text fig 1 as "ideal" curves. Theoretically, the divergence between the radial and ideal curves should increase with increasing tube length and decrease with increasing diameter. Qualitatively, the dependence on column length was verified by experiments which have been described. Up to a certain limit, depending on the dimensions of the tube, the divergence decreases as the angle between the axis of the tube and the radius of the rotor is increased.

After the motion of the ascending stream has become established the layer actually increases in width from the bottom of the tube to the boundary since sedimentation continues as the elements of fluid rise. The movement is opposed only by the viscous drag against the walls and against the body of the fluid. The same applies to the layer of particles deposited against the outside wall. Consequently, the most rapid movement occurs a small distance from the walls, and the body of the fluid below the boundary is made to exhibit a counterclockwise circulating motion by the action of these counteropposite

flows Immediately adjacent to the wall surfaces may be assumed to exist a very thin layer of bound fluid Particles deposited within this layer can move along the wall at only an infinitely low rate because of the enormous frictional drag As the material accumulates the newly arriving particles can move *toward the bottom of the tube more easily* In tubes inclined at small angles to the axis of rotation the component of force holding the particles against the wall is very large in comparison with that tending to move them, and it is not surprising that a "sticking" of large particles to the outer wall is frequently observed in such centrifuges As will be shown below, the effect for real particles subject to Brownian movements is more pronounced in high speed centrifuges than in low speed ones run sufficiently long to give a theoretically equivalent sedimentation

Ideal sedimentation in the angle centrifuge does not occur with small particles, since they are subject to the forces of diffusion There is backward diffusion of those particles collecting within the outer layer of bound fluid into the descending layer and from the body of the fluid into the ascending layer The latter is not cleared completely as with ideal particles but is only diminished somewhat in concentration Material is not deposited in a semisolid layer that slides down the outer wall but increases the concentration within an adjacent layer of fluid which then descends Because of diffusion there is a gradient of concentration across the descending layer, and the different portions attempt to settle to levels of corresponding densities within the body of the fluid At first a gradient of concentration is formed only at the bottom of the cell, but as the process continues each section of the gradient zone is expanded until a good portion of the column's height may be involved The actual deposition of material takes place only near the bottom of the tube

As regards the effect on the boundary movement, it can be said that the behavior of large particles in a high centrifugal field will naturally approach that of ideal particles When the diffusion rate is at all appreciable in comparison to the sedimentation rate, the boundary will be displaced less rapidly However, the boundary may be expected to progress faster in very dilute solutions than in concentrated ones, as reference to the schematic drawings of Text-fig 3 will show These represent the hypothetical distribution of concentration near some point along the inside wall of the tube, such as at *A*, Text-fig 2 For the ascending layer to rise at some given average rate, a certain difference in average density is required to be maintained between the layer and the body of the fluid In the present instance this difference is assumed to be equivalent to a difference in protein concentration of 0.1 per cent With a low concentration of material (hypothetical 0.23 per cent), considerable migration away from the wall is necessary to establish the required difference By the time the fluid now at *A* reaches the region of the boundary, which itself is diffuse, it may be almost cleared of protein and will seek the



TEXT FIG. 3. Schematic representation of the action within the ascending layer, which causes a lower relative concentration to be maintained within the layer as lower initial concentrations are used. Basis for the distributions of concentration shown is the assumption that a difference of 0.1 per cent protein is required between the ascending layer and the main body of fluid to sustain a given rate of movement. The third figure illustrates the process by which small amounts of a large protein can be carried above its respective sedimentation boundary.

upper edge of the boundary. The fluid now at *C* may be of the proper density to seek the central section of the boundary, with that at *B* rising to an intermediate level. Through the action of the ascending layer the slower portion of the boundary thus becomes more widely spread and the center of the boundary, or region of steepest concentration gradient, suffers a small additional displacement downward.

With a higher concentration (hypothetical 1 per cent), the same density difference (equivalent of 0.1 per cent protein) is reached much sooner, while the concentration at *A* is still not greatly different, proportionately, from that within the main body of the fluid. The ascending layer rises more rapidly than with the lower concentration, and most of the fluid, especially that now at *B* and *C*, seeks levels within the faster section of the diffuse boundary. There is little, if any, additional displacement of the boundary, but the leading edge of the boundary is progressively widened until a fair concentration gradient extends completely to the bottom of the tube.

It is interesting to consider the case in which a material  $\beta$  (Text-fig. 3) of low concentration and high sedimentation rate is centrifuged simultaneously with a smaller, more concentrated protein  $\alpha$ . A suspension of virus particles in a solution of serum albumin might furnish such an example. Two sedimentation boundaries will be formed, and if they could proceed according to normal sedimentation, they would in time become well separated with entirely indistinguishable amounts of protein  $\beta$  being found in the vicinity of the diffuse  $\alpha$  boundary. However, in the early stages of centrifugation, there is considerable overlapping of the two boundaries by reason of the fact that the rate at which a boundary diffuses or spreads is proportional to the square root of the time, in contrast to a linear relationship for the displacement by sedimentation. Because of the continual removal of partially cleared fluid by the ascending layer, this condition of overlapping is perpetuated, in a sense, along the inner wall of the tube. In the diagram at the right of Text-fig. 3 it can be seen that the levels of comparative density which will be sought by the fluid now between *A* and *B* correspond to positions within or near the boundary of protein  $\alpha$ . An appreciable quantity of  $\beta$  is carried along by this fluid into regions where it would not otherwise be expected. Of course, if  $\alpha$  has an extremely low sedimentation rate, the corresponding ascension to the region of diminished concentration will occur so slowly that almost all of the large, more rapidly moving particles will have time to migrate out of the ascending layer before it has progressed appreciably above the  $\beta$  boundary.

Neglecting the effects of convective disturbances, the imposition of a synthetic density gradient upon a solution or suspension of particles should cause the boundary movement to approach more nearly that exhibited in the ultracentrifuge. As an elemental volume within the ascending layer is partially or completely cleared of sedimentable material, it has to move only a relatively

short distance, depending on the strength of the gradient, before it reaches a region of comparable concentration

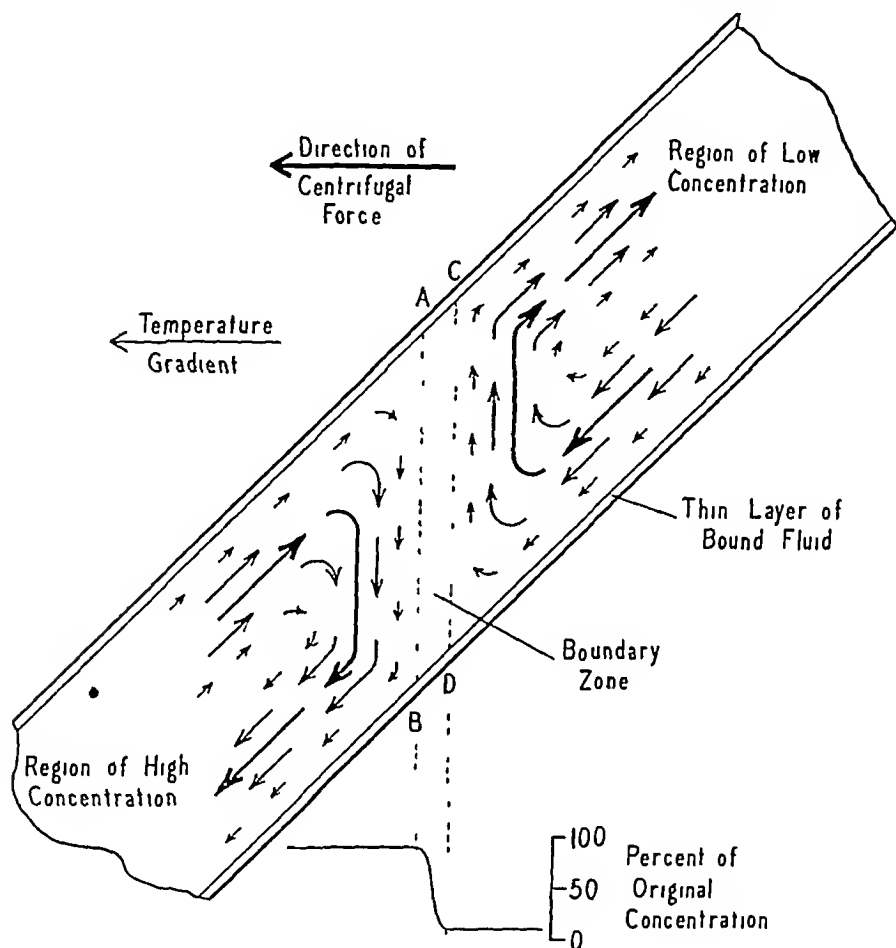
*Theoretical Considerations of Corrective Disturbances*—Even with angle centrifuges of the vacuum type, in which conditions are ideal as regards the absence of temperature disturbances, some remixing of partially sedimented material occurs during deceleration, as was shown experimentally. This is caused by the tendency of the fluid to continue the rotation. The liquid near the outer walls is decelerated at a more rapid rate, in linear measure, than that closer to the axis of rotation. Across the fluid along the radius exists a gradient of tangential decelerating forces which causes a torque to be exerted on the fluid. Thus a circulation is established in the same direction as the direction of the centrifuge's rotation. The viscous drag against the walls helps to slow down the circulation, but only an appropriate density gradient establishing a countering torque can prevent it. This torque is due to the tendency of the denser elements to remain near the outer wall of the tube. The magnitude of these forces which are opposed to the circulation is directly proportional to the centrifugal force, whereas the forces prompting circulation are directly proportional to the rate of deceleration. Hence it is important to decelerate a centrifuge especially slowly just before it stops, when the centrifugal force is very small. The action of a synthetic density gradient in minimizing stirring is immediately apparent. Incidentally, the gradient due to hydrostatic compression of the fluid offers appreciable assistance in reducing convective disturbances only in very high centrifugal fields (13). It can be shown that even then the only type of convection which can be completely inhibited is that which would otherwise be caused by a temperature gradient directed exactly parallel to the centrifugal field.

A centrifuge such as the electrically driven one (5) used in the present studies which spins in the open air or in any other gas is subjected to the frictional resistance of the gas, causing a generation of heat which must be dissipated into the same medium. The surface consequently tends to warm up to a certain equilibrium temperature which is higher for a more rapidly moving surface. As a result heat will be transferred from the outer to the slower moving inner sections of a rotor, and a temperature gradient toward the outer sections will be maintained.

Referring to Text fig 4 which represents a section of a centrifuge tube, consider as a simplification that the temperature gradient is parallel to the centrifugal force. An elemental volume of fluid in the outer portions of the tube, for example, is thus warmer and consequently less dense than the neighboring fluid located closer to the axis of rotation. This elemental volume tends to seek a new level nearer the axis of rotation where the density of the fluid is more comparable to its own. This tendency can be quite appreciable even with very small temperature gradients, since the buoyancy forces increase



directly with the value of the centrifugal force. The attempt of the fluid to reach a more stable state generally results in a circulating flow or convection. In the case of a water-filled ultracentrifuge cell with an imposed radial temperature gradient, the fluid would rise along the side walls and return with lowered



TEXT-FIG 4 Section of an inclined centrifuge tube illustrating how, during operation of an angle centrifuge spinning in the open air, convection currents above and below the boundary keep it abnormally sharp

temperature down the central portion of the cell. With an inclined cylindrical tube containing fluid in which a sedimenting boundary is present it is difficult to predict either the exact flow pattern or the distribution of temperature throughout the fluid although the temperature variation along the walls be known. However, the schematic drawing of Text-fig 4 may be used for purposes of argument.

Across a sedimenting boundary there is a comparatively rapid change of

and density with radial distance by reason of the gradient of solute concentration. Below the boundary, for example, any elemental volume of fluid seeking a new level of slightly different fluid density will find it necessary to rise only to the outer edge of the boundary if the concentration of sedimenting material is sufficiently high. It can be seen, as indicated in the drawing, that separate circulating flows are set up above and below the boundary. Whenever the concentration gradient at either edge of the boundary reaches a certain minimum value, i.e. when the difference between the density of circulating fluid near the boundary and the density of an adjacent layer within the boundary reaches a certain minimum value, the respective layer becomes incorporated in the circulation. Thus the boundary is kept abnormally sharp. The steep concentration gradients within the boundary orient themselves so as to furnish counter torque which prevents stirring that otherwise would be initiated within the boundary through the action of temperature gradients and shearing forces. Since there is a steep gradient of concentration through the boundary zone, there will be a continuous and relatively rapid diffusion of particles across the boundary into the less concentrated region. The convection currents above and below the boundary maintain uniform distributions of concentration in these regions.

Within the boundary zone the radial sedimentation of particles proceeds normally, and thus the boundary zone, the zone of high concentration gradient, is made to progress along the tube. However, its rate will not be characteristic of a normal radial sedimentation because of factors related to the accelerated diffusion across the boundary. The activity or strength of the circulating flow on either side of the boundary is determined in considerable degree by the height of the respective column of fluid. Because of the restraining action of the bounding surfaces and the increased interference offered by the counterflow, convection is reduced in short or narrow columns. For example, when the boundary reaches the lower section of a tube, the convection is more active above and less active below. Particles then diffusing across *AB* (Text fig. 4) to the boundary zone are not so readily replaced on the left of *AB* by the circulation, and the concentration in the vicinity of *AB* is decreased. The influence of this decrease is transmitted through the boundary, lowering the concentration at every point. When the gradient at *CD* is decreased below a threshold value by lowering of concentration within the boundary zone, the fluid there (near *CD*) is then able to join the circulating flow above the boundary. With this process in continuous operation the net result is an additional progressive displacement of the boundary along the tube. By a similar argument it can be shown that theoretically, as was actually observed experimentally, a boundary near the meniscus can be displaced toward the meniscus. However, in actual centrifugations this action appears to be subdued since observed rates have always been greater than the value for normal,

radial sedimentation This is possibly explained by the fact that whereas convection in the supernatant is comparatively uninhibited, interference below the boundary is offered by the directly opposing motion of the ascending and descending layers established by the sedimentation of particles close to the walls

A certain gradient of concentration must be maintained within the boundary zone to prevent a single circulatory system from being established for the whole column With a very low concentration of material, the distance between  $AB$  and  $CD$ , i e, the width of the boundary zone, must then become very small Since the gradient is kept at a minimum value, the same number of particles can be transported per second as with higher concentrations, but, because of the smaller total number of particles concerned, equal concentrations will be quickly established on both sides of the boundary, and the boundary will vanish

Where thermal convection is present, with or without boundary formation, sedimentation continues to take place but is theoretically never complete The more pronounced the convection and the lower the concentration of material, the slower the deposition of sediment at the bottom of the tube Particles migrate toward the layer of bound fluid along the outer wall, and those which do not diffuse back into the circulating fluid form aggregates or a denser layer of solution which is able eventually to reach the bottom of the tube As a first approximation sedimentation will be more or less logarithmic, during any given interval of time a certain fixed proportion of the particles still circulating within the fluid at that time will be deposited Concentration gradients are so steep in the vicinity of the sediment that little remixing into the fluid occurs

Thermal convection can be prevented by imposing on the solution a synthetic density gradient of some non-sedimentable material The strength of the gradient should be sufficient to counteract completely the opposing density gradient set up by the variation of temperature through the fluid

*Discussion of Practical Aspects* —From an investigation of a given material with an angle centrifuge, it is hazardous to draw any conclusions regarding normal sedimentation rate, particle size, or homogeneity unless the existence of a sedimentation boundary has been demonstrated For small tubes (1.5 cm diameter or less) in a properly operated centrifuge of the vacuum type, a very approximate semiempirical relationship could be derived for determining the amount of sediment in terms of normal sedimentation rate, provided the concentration of material were known to exceed a few tenths of 1 per cent However, a method based on measuring the displacement of a boundary has certain important advantages and is more general in its application It has been shown that tubes can be removed from a centrifuge, reoriented, and sampled or photographed without greatly altering a boundary Stirring during deceleration is more serious but can be rendered inconsequential by decelerating slowly just before stopping the centrifuge and by providing a sufficient opposing

density gradient, either synthetically or by the use of relatively concentrated material

From consideration of both experiment and theory, it can be said that the rate (measured radially) at which a boundary will migrate away from the axis of rotation in an angle centrifuge of the vacuum type is equal to or somewhat greater than the normal rate as measured in an ultracentrifuge. For tubes of ordinary dimensions the actual rate should never exceed the normal rate by a factor of as much as 2. The same generalities apply to centrifuges spinning in the open air, but they must be regarded with less certainty because of the thermal convection currents of unpredictable nature which have been experimentally demonstrated in such cases. For efficient separation in centrifuges of the latter type, tubes of the smallest practical bore should be used, although some compromise with capacity may be necessary.

A boundary, *i.e.*, the region where there is a relatively abrupt change in the concentration, may be located by optical means or by other tests made on samples taken carefully from the tube at different levels (3). A supernatant fluid relatively free of the material under study and a marked gradient of concentration between the boundary and the bottom of the tube are characteristic of optimum sedimentation in an angle centrifuge. However, there is ample physical basis to explain the presence of small amounts of a homogeneous material in the supernatant fluid. Although it may be impossible to reach definite conclusions regarding the relative particle size of such residual material, inferences perhaps can be drawn if results are compared with new determinations made after incorporating all the refinements which have been discussed.

If thermal convection has been very active, a uniform distribution of material will be found above the boundary and also in a higher concentration below the boundary. The boundary may be abnormally sharp. Incidentally, phenomena studied in the present work can be used to test, by a simple procedure, for possible thermal convection in the ultracentrifuge cell (10). In the first place unusually sharp boundaries should always be regarded with suspicion. If a slight misalignment of the cell from its normal position does not cause an additional concentration gradient to be introduced below the boundary it may be concluded that convection is present.

In designing angle centrifuges for purely preparative purposes one is usually interested in attaining the maximum possible efficiency, which may be defined as the ratio between the volume of fluid cleared and the equivalent time required at speed. Allowances are made for the time of acceleration and deceleration in figuring the equivalent time. As an approximation the efficiency may be considered as proportional to the product of the average centrifugal force, the total capacity of the centrifuge, and the reciprocal of the projected distance, measured radially, between the surface of the fluid and the outer edge of the tubes. Greatest efficiency with diffusible material can be realized with tubes oriented at small angles ( $10$ – $20^\circ$ ) to the axis of rotation. Centri

fuges with such tubes have been described by Masket (11), and a theoretical treatment of the stresses developed has shown them capable of high speeds in spite of large numbers of tube holes (12). However, small angles are not suitable for general clarification work with large non-diffusing particles, which will collect along the wall of the tube. Furthermore, special arrangements (11) to prevent overflow must be provided, or else space is wasted and tubes collapse easily, also the precision with which a boundary can be located is probably lower with small angles.

When a centrifuge is to be employed for general purposes, including rough analysis or rapid clarification, angles near  $35^\circ$  and tubes of 1.3 cm bore have been found to represent a satisfactory compromise between efficiency and other factors. If particles of molecular weight below 100,000 are to be studied, centrifugal forces of at least 200,000 gravity should be employed in order that the boundary may clear the meniscus within a reasonable time (3, 12).

Convective disturbances of all types can be almost completely eliminated by imposing upon the liquid under study a synthetic gradient of sucrose or some other material of low molecular weight. In the present investigation, the gradients were prepared in a very rough uncertain manner and perhaps inadequately in some cases. It is believed that a great improvement might be had by preparing several samples of the material, each incorporating a few per cent more of sucrose, for example. These could be introduced into the tube at the bottom, starting with the least dense, and the tube allowed to stand for a time to permit a relatively uniform gradient to be established by diffusion. With a well formed and sufficiently steep gradient the boundary of a homogeneous material should progress at very nearly the normal (ultracentrifugal) rate, measured radially. Of course, the increased viscosity of the fluid over the section traveled must be taken into account and can be determined by measurements upon samples taken after the run. Furthermore, the technique can be applied to preparative procedures which have produced unsatisfactory concentration of a particular biological agent, for example.

Where convection has been minimized the following approximate formula can be used either for roughly estimating the particle size, assuming no extreme deviation from a spherical shape, or for approximating the time required to complete a sedimentation of nearly spherical particles whose physical properties are known

$$T = 54 \left( \frac{D - L}{D + L} \right) \left( \frac{\eta}{d^2(\sigma - \rho) S^2} \right)$$

where  $S$  is the rotational speed in R P M,  $L$  is the radial distance in centimeters from the meniscus to the axis of rotation,  $D$  is the radial distance from the boundary (or outer edge of tube for complete sedimentation) to the axis,  $T$  is the time in minutes,  $\rho$  and  $\sigma$  are the densities in grams per cubic centimeter of the medium and the particles, respectively,  $\eta$  is the viscosity of the fluid in

poises,  $d$  is the average diameter of the particle in centimeters. If  $\theta$  is the angle between the tube and the axis of rotation, then for a boundary displaced  $\lambda$  cm. along the tube from the meniscus,  $D = X \sin \theta$ . As an approximation of average conditions with aqueous solutions the formula can be reduced to

$$T = \frac{1.8}{d^2 S^2} \left( \frac{D - L}{D + L} \right)$$

where the viscosity has been taken as 0.01 poise and the fluid and particle densities as 1 and 1.3 gm. per cc., respectively

#### SUMMARY

1 Using hemocyanin from *Limulus polyphemus* as a test material, the process of sedimentation in the angle centrifuge, operating both in vacuum and in the open air, has been investigated.

2 Sedimentation in a given field of force was found less efficient when centrifugation was conducted in the open air, because of thermal convection.

3 Correlations have been made with results obtained in the analytical ultracentrifuge, and a theory of sedimentation in inclined tubes has been presented to explain the experimental results.

4 It has been shown that under proper conditions the angle centrifuge may be used for approximate determinations of particle size.

5 Recommendations, based mostly on experimental evidence, have been made for improving sedimentation and interpreting results.

6 To counteract convective disturbances of either thermal or inertial origin, a satisfactory method has been developed which consists of furnishing the fluid under study with a synthetic density gradient, formed with sucrose or some other non-sedimentable material.

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## EXPLANATION OF PLATES

## PLATE 1

FIG 1 Pictures *a* to *f* illustrate the effect on artificial boundaries of *Limulus* hemocyanin of acceleration to and immediate deceleration from 16,200 R.P.M. All boundaries were made to the same level as that of unspun sample *a* and allowed to stand for 135 minutes. Pictures *g* to *p* show the results of centrifugation at the above speed in angle centrifuges, except for *l*, which was obtained with the analytical ultracentrifuge. Equivalent centrifuge time *h* to *p*, 135 minutes, *g*, 270 minutes. Vacuum centrifuge *c*, *f*, *m*, *n*, others in open-air centrifuge. Concentration of 0.2 per cent, *a*, *b*, *c*, 0.8 per cent, *d*, *e*, *f*, *g*, *o*, *p*, 0.36 per cent, *j*, *k*, *l*, *m*, *n*, 1.8 per cent *h* and *i*. Pictures *i*, *k*, and *n* illustrate improvement of boundaries by addition of synthetic density gradient. In the photographic negatives the boundary of *o* appears blurred with the average position close to level indicated.

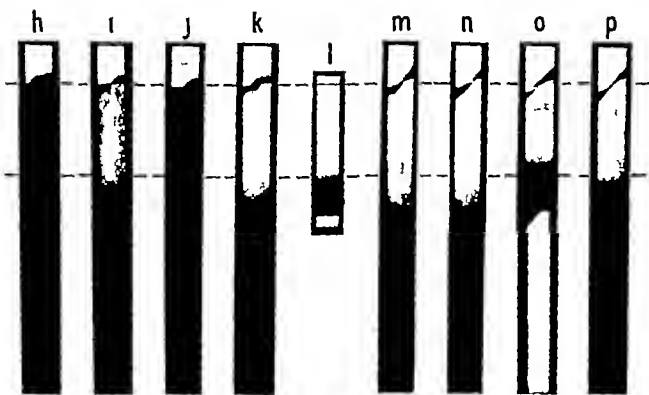
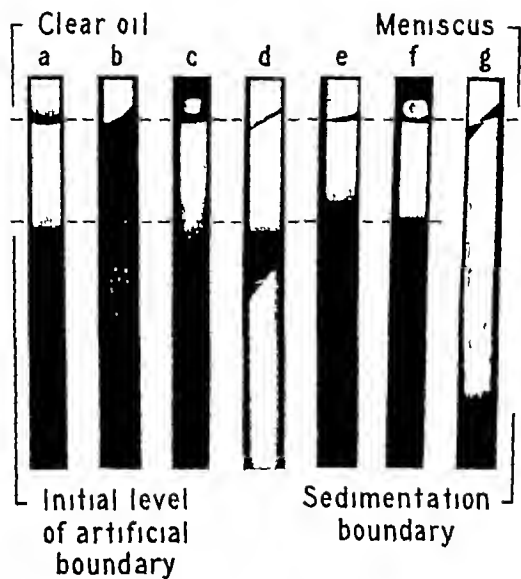


FIG. 1



## PLATE 2

FIG 2 Serial absorption photographs (*q* and *r*) comparing the sedimentation of 0.36 per cent hemocyanin in a normally oriented ultracentrifuge cell (*q*) with that in the same cell after a  $65^\circ$  misalignment (*r*). Pictures *s* to *w* represent experiments similar to those of Fig 1, except that the concentration is only 0.12 per cent. Vacuum centrifuge, *s* and *t*, open-air type, *v* and *w*. Synthetic density gradient of sucrose was provided in *t* and *w*.

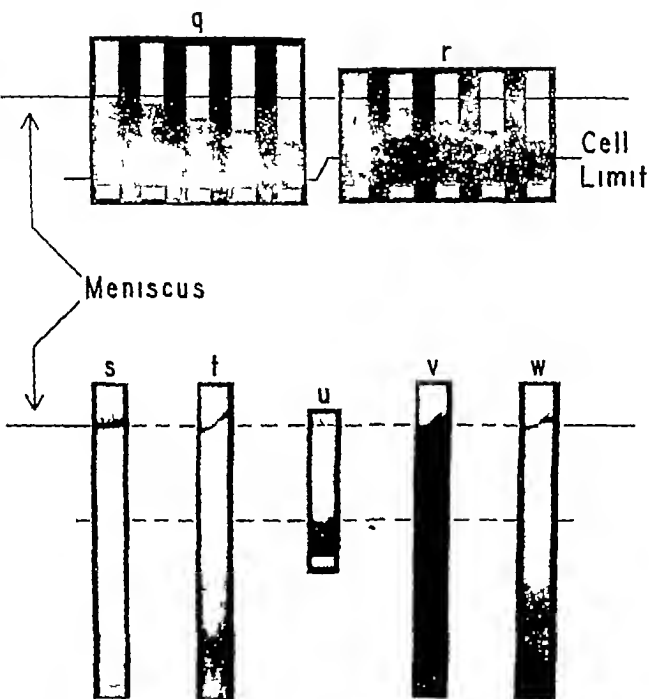


FIG 2

(Pickels Sedimentation in the angle centrifuge)

### PLATE 3

FIG 3 Photographs taken by a refractive index method and showing sedimentation at corresponding times in normal (*A*, *C*) and misaligned (*B*, *D*) ultracentrifuge cells. *C* and *D* correspond to the fourth pictures in sets *q* and *r*, respectively, of Fig 2. The concentration of hemocyanin in cases *A* and *B* was only 0.04 per cent.

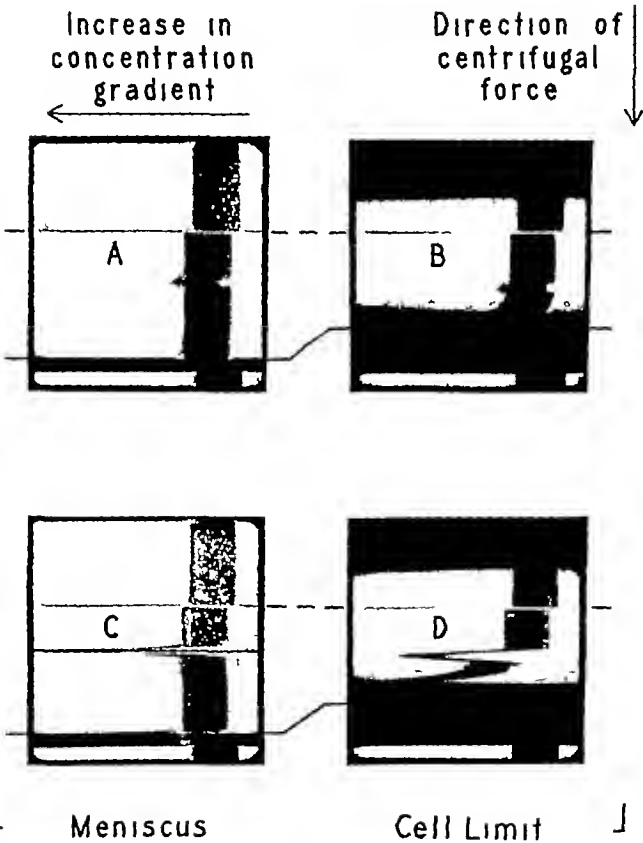


FIG 3

(Pickels Sedimentation in the angle centrifuge)



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## DERIVED PHOTOSENSITIVE PIGMENTS FROM INVERTEBRATE EYES

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(Received for publication, October 29, 1942)

Although most animals and plants are photosensitive, chemical data about visual pigments are restricted almost entirely to the visual purples of vertebrates (For a recent review, see Hecht, 1942) These substances are bleached by light and regenerate in the dark. Their absorption spectra correspond to the spectral luminosity function of the organisms (Koenig, 1894, Hecht, 1937, Wald 1937)

Many invertebrates possess excellent eyes, some, like the squid, have eyes which contain all the important features of the vertebrate eye, including a retina composed of rod like cells containing a clear red pigment and migratory black pigment granules. It is natural to extend the analogy of the vertebrate eye to the red pigment of the cephalopod retina by assuming it to be a light sensitive pigment like the vertebrate visual purple.

Krukenberg (1882) challenged this analogy in his report that light has no effect on the squid pigment. His observation has been confirmed by Wald (1941) and others, but was rejected by Hess (1905) on the ground that the melanin in the intact retina masks the bleaching. This difficulty, according to Hess, could be avoided by inducing opacity of the retina with formalin.

Re-examination of this problem showed that both positions are correct. Following Hess' procedure, a dark adapted squid retina was cut in half and immersed in 10 per cent formalin. No change occurred in the portion kept several hours in the dark, but it was astonishing to note that the part brought into the rays of the microscope lamp bleached in less than 5 minutes from bright red to dull gray. Obviously formalin had rendered the pigment photosensitive.

The red pigment may be brought into solution by means of saponin. Two retinas from a large squid are placed in 4 cc. of 4 per cent saponin solution. After 4 to 8 hours the insoluble residue is removed by centrifugation and the brownish red supernatant removed for study. The solution behaves like the original retina. If it is illuminated for 1 hour by a 100 watt projection bulb at a distance of 6 inches through a water filter, no change in its color or photometric density can be detected. If however, formalin is first added to make a concentration of 10 per cent, the same light causes an unmistakable bleaching.

Essentially the same results are obtained if another denaturant, like 10 per cent ethyl alcohol, is used instead of formalin. The solution decolors on exposure to light.



The quantitative changes which these solutions undergo in the visible spectrum have been studied by means of a photoelectric spectrophotometer. The apparatus includes a monochromator, the light from which passes through a 1 cm double cell mounted on a shuttle, and impinges on a rubidium photocell. A one stage amplifier is controlled by a potentiometer reading directly in per

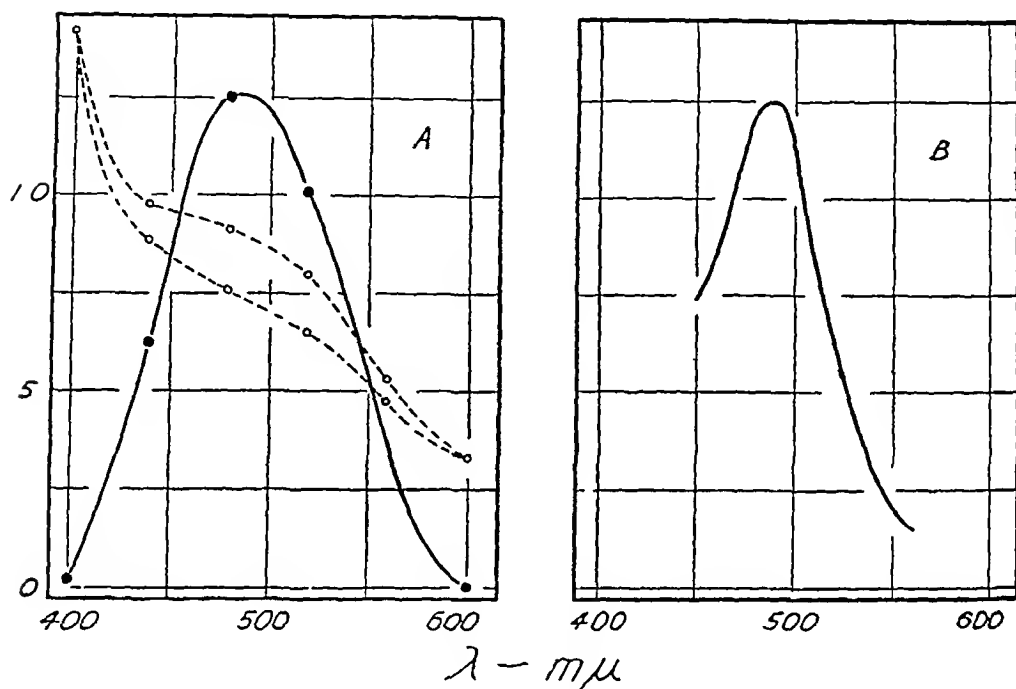


FIG 1 Spectral sensitivity of squid pigment. In A the broken lines show the density spectra before and after bleaching of an extract of a squid retina in 2 cc 4 per cent saponin brought to 10 per cent ethanol, while the continuous line shows the 10 times density difference spectrum due to illumination of the same solution. In B is given Piper's curve for spectral sensitivity of *Eledone*, recalculated for equal energy spectrum and equal response.

cent transmission. Results are expressed as photometric density according to the equation  $D = \log (I_{\text{incident}}/I_{\text{transmitted}})$ .

Measurements are made in accordance with either of two procedures. If the density of the experimental solution is desired, it is placed in one side of the cell, and the solvent in the other. If the effect of light is to be determined, the blank contains the same solution as the experimental. This method has the virtue of cancelling out changes not due to light, and permits accurate measurement of changes in regions of high density.

Fig 1A shows the absorption spectrum of one such saponin solution containing 10 per cent alcohol before and after its exposure to light, as well as the

difference between the two due to the bleaching action of the light. The difference spectrum is an approximately symmetrical curve whose maximum is at  $480\text{ m}\mu$ . The same maximum occurs with formalin treated solutions.

It is significant that the absorption curve of the artificially rendered photo-sensitive material is similar to the spectral sensitivity of the squid's eye. The best available data are those of Piper (1904) who measured the magnitude of the retinal electric response given by *Eledone moschata* in the spectrum of a Nernst lamp. Data of this type do not constitute a sensitivity curve because of unequal energy distribution of the Nernst lamp spectrum, and the lack of knowledge of the relation between response and energy. However, a rough approximation may be made first by correcting the data in terms of Pflüger's (1902) measurements of the spectral energy curve of a Nernst lamp, and second, by using Chaffee and Hampson's (1924) formulation of the relation of the frog retinal response to energy at low levels of response. This states that  $R \sim (I)^{1/2} a$ , where  $I$  is the relative energy of the Nernst lamp at a given wavelength, and  $a$  is the spectral sensitivity. The spectral sensitivity curve computed in this way is in Fig. 1B and shows a rough agreement with the bleaching spectrum in Fig. 1A. Closer agreement should not be looked for until the spectral sensitivity of the squid eye is measured in terms of the energy required for equal response in the spectrum.

The squid is not unique in the possession of a pigment which becomes light sensitive after formalin treatment. So far I have examined only the blue soft shelled crab *Callinectes hastatus*, and the horseshoe crab, *Limulus*, in spite of their taxonomic distance from the squid they contain similar pigments which become light sensitive on treatment with formalin. The data are shown in Fig. 2.

The response of an extract depends somewhat on the treatment. Thus, the prolonged bleaching of the formalized pigment of the crab results in a secondary maximum at about  $590\text{ m}\mu$ , as shown in Fig. 3. Another anomaly is shown by extracts of dried squid retinas, in which bleaching is maximal at  $455\text{ m}\mu$ . One extract of fresh squid eyes showed an interesting resemblance to vertebrate visual purple in that after bleaching it regenerated in the dark to within 50 per cent of its original concentration.

These formalin-sensitized light sensitive pigments should not be confused with the melanin like pigments found in insects and also in the squid. Thus the red screening pigment in the retinal sheath cells of *Drosophila*, when treated with formalin does not bleach in light and neither does the melanoid pigment from the squid eye.

Escher Desrivieres, Lederer, and Verrier (1938) have purified such a red pigment extracted from squid retinas by dilute alkali. Examination of retinas treated in this manner shows clearly that the great mass of the pigment they studied is composed of dissolved melanin granules. Its properties are dis-

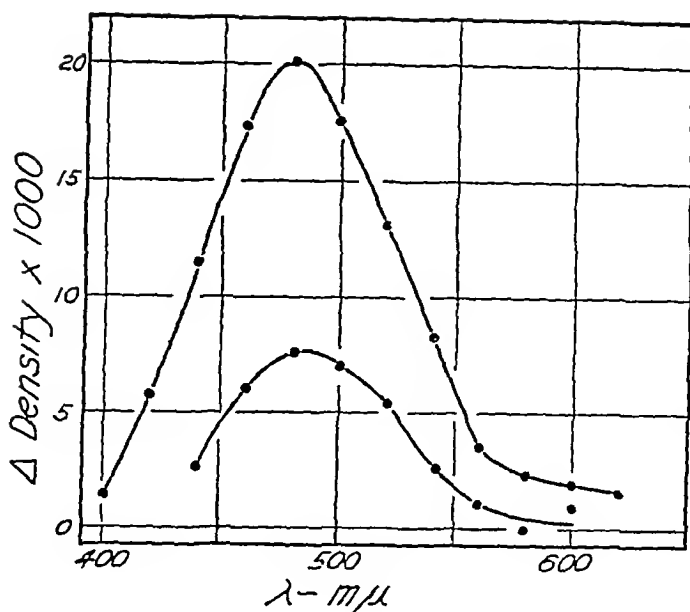


FIG 2 Absorption spectra of derived retinal pigments The upper curve is the difference spectrum for *Callinectes* derived from one retina dissolved in 1 cc 4 per cent saponin, and brought to 10 per cent formalin The lower curve is 2 times the difference spectrum for *Limulus* derived from 15 retinas of 4 cm wide animals, dissolved in 3 cc 2 per cent alkaline digitalin, and brought to 10 per cent formalin

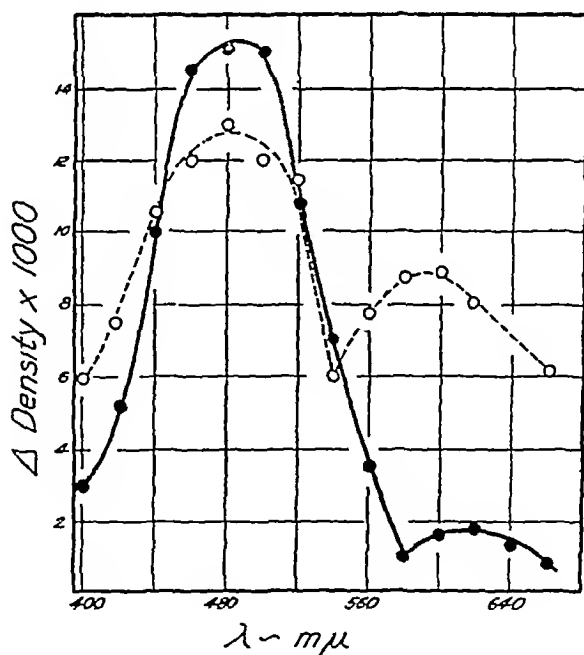


FIG 3 Prolonged bleaching of *Callinectes* pigment The continuous curve gives the difference spectrum due to the 1st hour of bleaching, while the broken curve gives the difference spectrum due to the 2nd hour of bleaching

tinctly different from those of the homogeneously distributed red pigment of the cephalopod rods. The granular pigment is stable in the presence of acid and alcohol, and is insoluble in detergents, the homogeneously distributed pigment is destroyed in the dark by acid, and in the light by alcohol and formaldehyde, and is extractable by detergents like saponin and digitonin, used to dissolve visual purple.

If the residue from a saponin extraction of the squid's eye is treated with dilute alkali, a magenta pigment is obtained whose absorption spectrum shows

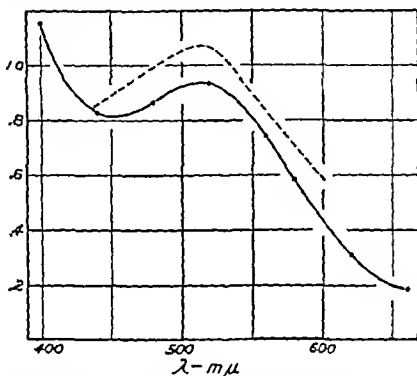


FIG 4 Absorption spectra of non light sensitive pigments. The continuous curve is the absorption spectrum of the residue from a saponin extract of squid retina, dissolved in 0.1N NaOH. The broken curve is the absorption spectrum of the "prosthetic group" from squid retinal chromoprotein, according to Escher Desrivieres, Lederer and Verner

a maximum at 510 m $\mu$ . An example is in Fig 4. This pigment may be related to the "prosthetic" group of Escher-Desrivieres, Lederer and Verner, obtained by hot acid hydrolysis from their 95 per cent phenol soluble "chromoprotein". However, it is unaffected by light in the presence of formalin.

In its bleaching by light, the formalin-sensitized pigment shows some chemical similarity to visual purple because it liberates retinene. Retinene was first found in the squid eye by Wald (1941) who showed that, although the total potential concentration of retinene in the fresh squid retina is high (which I can confirm), only about 10 per cent of it is released by exposure to 45 minutes of daylight. In the present experiments retinas first treated with formalin release large quantities of retinene on illumination.

Retinas from freshly decapitated animals were removed immediately in daylight to filter paper and dried in a vacuum desiccator. They were then shaken for 30 minutes with petroleum ether to remove free carotenoids. A pair of weighed retinas was soaked 5 minutes in water and placed in 10 per cent formalin for 15 minutes. One retina was illuminated 15 minutes by a 100 watt projection bulb at 6 inches distance through 3 inches of water and a yellow filter (Corning 368 half thickness) to protect the liberated retinene from photic destruction. The second retina served as a dark control, but otherwise received similar chemical treatment. After washing for 5 minutes and then drying, the retinas were again shaken with petroleum ether for 30 minutes.

TABLE I

*Retinene Production in the Retina of the Squid*

The figures give the photometric density of retinene in  $\text{CHCl}_3$ , at 390  $\mu$ , per mg dry weight of retina

Treatment	Extraction	Retinene concentration	
		Retina in light	Retina in dark
First illumination, 15 min	1	0 0060	0 0031
	2	0 0051	0 0016
	3	0 0027	0 0017
	4	0 0019	0 0008
	5	0 0007	—
	Total	0 0164	0 0072
Second illumination, 15 min	1	0 0008	0 0031

Each solution was evaporated to dryness and the residue taken up in 2 cc of chloroform for spectrophotometric analysis. Table I gives the retinene content of six successive petroleum ether extractions of the same pair of retinas. After all the retinene was extracted, the retinas were again treated with formalin and extracted. It is evident that considerable retinene is released by the formalin treatment itself, but that an illumination of 15 minutes releases nearly all of the remaining retinene.

Just how related all this is to the vision of the squid, it is hard to say. It may merely be that the normal squid photopigment is relatively light-stable, and that the formalin treatment renders it light unstable. In that case serious consideration must be given to the possibility that the bleaching of vertebrate visual purple is a specialization and that the absence of bleaching, as in photosynthesis and photodynamic action, may have no direct bearing on the efficiency of a visual pigment.

## SUMMARY

The red pigment in the eyes of the squid, blue crab, and horseshoe crab becomes photosensitive when treated with formalin, and bleaches in the light. The resulting change in density is approximately symmetrical around a maximum at 480 m $\mu$  in the blue green. This difference absorption spectrum is in rough agreement with the spectral sensitivity of the cephalopod eye and differs only slightly from the difference absorption spectrum of vertebrate visual purple.

The formalin-sensitized pigment is not melanoid. Its bleaching in squid retinas releases large quantities of retinene.

It is suggested that the light sensitivity of the normal squid photopigment may be independent of its light stability.

I am pleased to acknowledge the kind advice and encouragement of Professor Selig Hecht.

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# THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

## VI. THE PROTAMINE COLLODION MEMBRANE, A NEW ELECTROPOSITIVE MEMBRANE

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(Received for publication, November 11, 1942)

### I

Previous publications<sup>1, 2</sup> in this series have dealt with the preparation and electrochemical properties of the activated (electronegative) collodion membrane. The activation consisted of an oxidation of the nitrocellulose, a process which caused an increase in the number of dissociable groups in the pores of the membrane. Such membranes show more pronounced effects than similar untreated membranes when tested by electrochemical methods such as the extent of anomalous osmosis, the magnitude of concentration potentials, and the rate of electroosmosis. The purpose of the present communication is to present a method for the preparation and a description of electropositive membranes which are analogous in most respects to the activated collodion membranes.

The literature on electropositive membranes has recently been reviewed by Manegold and Kalauch.<sup>3</sup> Two general methods for the preparation of such membranes are available. First, they have been prepared from inherently electropositive materials, such as the oxides of aluminum, zirconium or thorium.<sup>4, 5</sup> The second method consists of impregnating membranes prepared from collodion or similar materials with some basic organic compound.

One objection to the use of the polyvalent metal oxide membranes is that the electropositive charge is rather easily reversed in the presence of many electrolytes. For this reason they are not suitable for most purposes.

The impregnated membrane, in many instances, has proven more practical. The impregnation of collodion with basic substances may be accomplished either by dissolving such substance in the collodion solution or by adsorbing the material on the surface of a previously cast membrane. Among the

<sup>1</sup> Sollner, K., and Abrams, I. *J. Gen. Physiol.*, 1940, 24, 1

<sup>2</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, 25, 7

<sup>3</sup> Manegold, E., and Kalauch, K., *Kolloid Z.*, 1939, 88, 257

<sup>4</sup> Prausnitz, P. H. and Reitschötter, J., *Electrophoresis, Elektroosmose, Elektrodialyse*, Dresden: Theodor Steinkopff, 1931

<sup>5</sup> Sollner, K., and Liapountzeva, T. unpublished



ether-alcohol soluble substances thus far employed are basic dyestuffs<sup>6-8</sup> and alkaloids<sup>9, 7</sup>. Dried membranes prepared from such solutions have on occasions given concentration potentials as large as  $-50$  mv<sup>7</sup>. A disadvantage of this method, however, is that in aqueous media the basic substance slowly diffuses from the structure, and in time the membrane again becomes negative. This is the case with the dried membrane, and as might be expected, the loss of the impregnated material is even more rapid with the porous membrane<sup>10</sup>.

The adsorption method which has been used in the production only of *porous* electropositive structures consists of adsorbing a protein on the surface of a previously cast porous collodion membrane. Loeb,<sup>11</sup> for example, employed gelatin, egg albumin, casein, and oxyhemoglobin. Such membranes retain their characteristics practically indefinitely when kept in water. However, the proteins which have been employed are cationic only within restricted limits, *i.e.*, on the acid side of their isoelectric points. Thus membranes prepared with the more common proteins are electropositive only in distinctly acid solution. The preparation of dried membranes by this method has not been reported in the literature.

For the preparation of porous electropositive membranes, we have also employed the adsorption method, but with a protein having an isoelectric point well on the alkaline side of neutrality. Electropositive membranes which correspond in their properties to the dried collodion membrane can be prepared in a similar way by a modification of the adsorption method, as will be shown below.

The protein employed in this investigation was a protamine. Protamines are obtained from ripe fish sperm. They have an isoelectric point of 12.0 to 12.4,<sup>12</sup> and thus are cationic not only in acid, but also in neutral and even in weakly alkaline solution. They have a molecular weight of 2000-4000<sup>13</sup> and are decidedly water-soluble. Because of their basic character, the protamines have a tendency to combine with acidic substances. For these reasons, they seem ideally suited for our purpose.

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<sup>7</sup> Wilbrandt, W., *J. Gen. Physiol.*, 1935, **18**, 933.

<sup>8</sup> Nakagawa, J., *Jap. J. Med. Sc., III Biophysics*, 1937, **4**, 343.

<sup>9</sup> Harkewitsch, N. K., *Kolloid-Z.*, 1929, **47**, 101.

<sup>10</sup> Several experiments of this nature were carried out and it was found that porous membranes prepared from a collodion solution containing a basic dyestuff or alkaloid retain electropositive characteristics for a period not exceeding 24 hours.

<sup>11</sup> Loeb, J., *J. Gen. Physiol.*, 1920, **2**, 577, and many later publications.

<sup>12</sup> Schmidt, C. L. A., *The chemistry of the amino acids and proteins*, Springfield and Baltimore, Charles C. Thomas, 1938, 618.

<sup>13</sup> Gortner, R. A., *Outlines of biochemistry*, New York, John Wiley and Sons, 2nd edition, 1938, 465.

## II

The general method, as was indicated above, consists of the adsorption of protamine from an aqueous solution onto the surface of a previously cast porous collodion membrane. Membranes of high activity can be prepared by either of two methods. The one consists of an activation of the collodion membrane (by oxidation) previous to immersion in a solution of salmine sulfate.<sup>14</sup> The other method consists merely of an adsorption of the protein from an isoelectric (pH about 12) protamine solution.

Membranes were cast in test tubes (about 30 X 100 mm.) from a 5 per cent solution in ether alcohol (75-25) of Baker, U.S.P., collodion. This solution was poured slowly from the tube, the latter being constantly rotated. After the solution ceased to drip from the tube the solvent was allowed to evaporate for 2 to 3 minutes with the tube in an inverted position,<sup>15</sup> after which time it was immersed in distilled water. The resulting membrane was loosened from the tube and attached by the open end to a glass ring. Following a thorough washing to remove the excess solvent, the membranes in the case of the first method are oxidized with 0.5 N sodium hydroxide.<sup>2</sup> After another washing they are immersed for at least 12 hours in solutions containing 0.5 to 0.8 gm. of salmine sulfate per 100 ml. of solution. Finally, they are removed from the solution, washed and kept in distilled water.

If the other method is employed, the oxidation step is omitted and the protamine solution used for the adsorption is first brought to its isoelectric point.<sup>16</sup> This may be accomplished either by adding a stoichiometric amount of barium hydroxide to the sulfate solution or by simply adding sufficient sodium hydroxide to bring the pH of the solution up to 12.0.<sup>17</sup> Otherwise, the process is identical.

Actually it is unnecessary to use concentrations of protamine as high as those men-

<sup>14</sup> We are indebted to Eli Lilly and Company of Indianapolis who kindly provided us with samples of this material.

<sup>15</sup> This technique differs slightly from that employed previously in that formerly the solvent was allowed to evaporate with the tube in an upright, somewhat inclined position. The resulting membranes were always considerably less dense near the bottom since the solvent evaporation was retarded at the bottom and hastened at the open end. The present technique allows the downward escape of the solvent vapors, and hence the porosity in various parts of the resulting membrane is more nearly the same. In all likelihood, the over all structure of this membrane is similar to that of the middle section of the membrane used in former work. In addition to being more homogeneous (macroscopically) the present membranes are less likely to tear at the closed end.

<sup>16</sup> This method was employed by Hitchcock (Hitchcock, D., *J. Gen. Physiol.*, 1925, 8, 61) who prepared gelatin- and egg-albumin-collodion membranes.

<sup>17</sup> Whether this second method is basically different from the first is open to some question. The alkalinity of the isoelectric solution undoubtedly brings about some oxidation of the collodion which would result in a strong adsorption of protamine. It is thus possible that the adsorption process in these alkaline solutions is enhanced by a simultaneous oxidation-activation of the collodion.

tioned, i.e., 0.5 to 0.8 per cent solutions. Maximum activation can be obtained with solutions having a concentration of 0.1 gm per liter. The more concentrated solutions were used to prevent the exhaustion of the protamine since each solution was employed repeatedly.

Membranes prepared by this method have retained their original electro-positive characteristics for a 12-month period, bacterial contamination being prevented by the addition to the water of small quantities of thymol. Indications are that with proper precautions, these properties can be maintained indefinitely.

### III

In dealing with the activated collodion membranes, we have employed<sup>1, 2, 18</sup> three methods for establishing the electrochemical activity of a given porous membrane. These consist of determining (1) the characteristic concentration potential across the membrane, (2) the rate of electroosmosis through the membrane, and (3) the extent of anomalous osmosis. Of these methods, the last is able to distinguish most decisively between specimens having only slightly different electrochemical characteristics. For this reason it has been used extensively in this series of investigations on the structure of the collodion membrane and is used also for the characterization of porous protamine-collodion membranes. For the details of the three methods mentioned, their merits, and their theoretical foundations, the reader is referred to the previous publications. The method used in determining the extent of anomalous osmosis is described below.

Any arbitrarily chosen electrolyte could be used for this purpose. To obtain decisive, characteristic, and easily reproducible effects, a dilute solution of an electrolyte having a divalent ion identical in the sign of its charge with the electrokinetic charge of the membrane is employed. Negative membranes were tested<sup>2</sup> with a salt having a univalent cation and a divalent anion, such as potassium sulfate. Correspondingly, with positive membranes, a di-univalent salt is used, calcium chloride being used in this investigation. With the uni-univalent salts, the effects are not nearly as pronounced. On the other hand, with salts having a trivalent ion, such as aluminum, the latter often adsorbs on the surface of the membrane and so unduly influences its electrochemical behavior.

In testing positive membranes, therefore, the following technique is employed. The membrane bag attached to a glass ring is filled with a solution of 0.01 M calcium chloride and is attached to a rubber stopper holding a capillary manometer tube (inner diameter about 1.6 mm). The rate of anomalous osmosis is determined by measuring the rise of the meniscus in this tube 20 minutes after the filled membrane had been

<sup>18</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, 24, 467.

immersed in a large volume (about 600 ml.) of distilled water. This rise of the meniscus is taken as an indication of the "activity" of the membrane tested.

The electrochemical behavior of the proteized membrane varies with the ionic state of the adsorbed protein; therefore, one of the most important points was to determine the dependence of the behavior of protaminized membranes upon pH. Experiments were therefore performed to determine the relationship between anomalous osmosis and pH. A very active protamine-collodion membrane was filled with 0.01  $M$  calcium chloride and tested in the manner described; the pH of the inside and outside media was adjusted to the value desired by the addition of sodium hydroxide or hydrochloric acid. The results

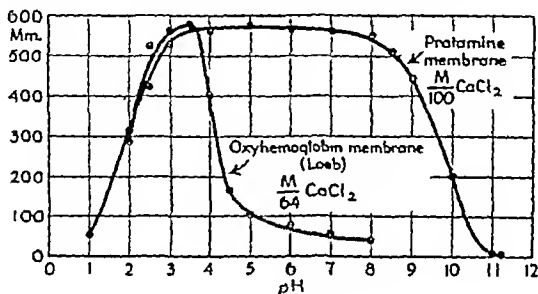


FIG. 1

are shown graphically in Fig. 1, pH being plotted on the abscissa and the values for the manometric rise after 20 minutes on the ordinate.

Similar experimental data obtained by Loeb<sup>11</sup> with an oxyhemoglobin-collodion membrane have also been plotted in Fig. 1. As is obvious, the range of pronounced electropositive activity in this case lies between pH 2 and 4, whereas the protamine membrane exhibits optimum effects between pH 3 and 8, the activity being depressed only in extremely acid and alkaline regions, distinct electropositivity remains evident down to a pH of 1 and up to 10. The exact magnitude of the effects at optimum pH obtained with the protamine and the oxyhemoglobin membrane are not strictly comparable since Loeb used  $M/64$  solutions whereas 0.01  $M$  solutions were employed in the other case.

In a previous publication,<sup>1</sup> the typical  $\Lambda$  shape of the liquid transport curves of Loeb were reproduced with an unoxidized and an oxidized collodion membrane. The electrolytes employed were the chloride, sulfate, and citrate of potassium. For the sake of comparison with regard to both the concentration effect and the general shape of the curves, we carried out analogous experi

ments with an active protamine-collodion membrane, the salts employed in this case being potassium, sodium, calcium, and cerous chlorides. In every case, the neutral salt solution was used. The results of these experiments are shown in Fig. 2. The osmotic behavior of the membranes with non-electrolytes is

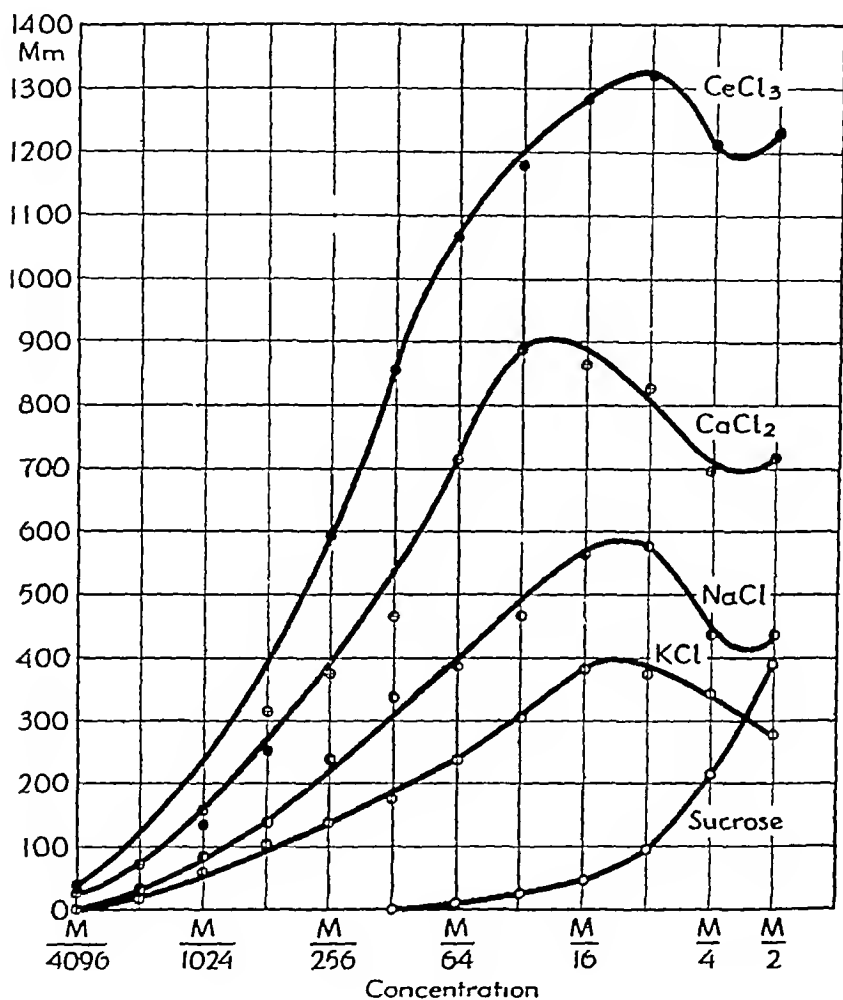


Fig. 2

indicated by the sucrose curve. The manometric rise of the meniscus, representing the rate of transport from the side of the water to the side of the solution, is plotted on the ordinate, with concentration (on a logarithmic scale) on the abscissa.

The curves of Fig. 2 assume the same general shape as those for the electro-negative membranes. Even the magnitude of the manometric rises with potassium, calcium, and cerous salts, respectively, are exceedingly similar to those

obtained with the oxidized collodion membrane using potassium chloride, sulfate, and citrate. Thus, we can say that with respect to its anomalous osmotic activity, the porous protamine-collodion membrane is an electropositive counterpart of the porous activated collodion membrane described previously.<sup>1 2</sup> We might add that the transports with the protamine membranes in neutral solution are considerably greater than those reported by Loeb for gelatin-, egg-albumin, casein, or oxyhemoglobin collodion membranes in acidified salt solutions.

Negative osmosis, a transport of fluid through a membrane from a concentrated to a dilute solution according to theoretical considerations of Sollner,<sup>19</sup> can be expected only under specific experimental conditions. Generally, with sufficiently porous membranes, if the faster diffusing ion of the electrolyte involved has the same sign of charge as that of the fixed ionic layer of the membrane, negative osmosis can occur. With the porous protamine membrane, then, this phenomenon can be expected to be most pronounced with solutions of strong acids.

In carrying out the experiments, the procedure of Loeb<sup>20</sup> was followed. The membrane filled with distilled water was immersed into acid solutions varying in concentration from  $M/1024$  to 2  $M$ . The rise of the meniscus in 10 minutes<sup>21</sup> representing the flow of liquid from concentrated to dilute solution, is plotted on the ordinate in Fig. 3 with the acid concentration on the abscissa on a logarithmic scale as before. Negative values indicate that the net fluid transport is from solution to solvent.

The striking feature of the curves in Fig. 3 is that negative osmosis is predominant at relatively high concentrations in comparison with those concentrations at which positive anomalous osmosis occurs.

Without entering into the more theoretical aspects of this matter, it might be well to bring out the fact that initial potential differences across such systems as are represented in Fig. 3 are quite high. For example, in the system 1  $M$   $H_2PO_4$  [membrane|water] the initial potential difference was found to be +180 mv.

Ordinarily, electroosmotic phenomena are absent or very weak in high concentrations of strong electrolyte solutions. Thus the ascription of negative osmosis to electroosmotic forces might raise some question. However, we must consider that in our experimental arrangement, a steep concentration gradient exists within the membrane between the solution on the one side and the water (initially) on the other

<sup>19</sup> Sollner, K., *Z. Elektrochem.* 1930 35, 36, 1930, 35, 234, Sollner, K., and Grollman A. *Z. Elektrochem.* 1932 35, 274 Grollman, A., and Sollner, K., *Tr. Electrochem. Soc.*, 1932, 61, 487

<sup>20</sup> Loeb J. *J. Gen. Physiol.*, 1919, 2, 173

<sup>21</sup> The duration of these experiments was only 10 minutes (as compared to 20 for the positive anomalous osmosis tests) since the initial rate of rise in this period is much more significant than the average rate over a longer period.

The active electroosmotic process would necessarily occur at such layers of the membrane in which the conditions are optimal for their occurrence,

This deduction is substantiated by the observation that negative osmotic flows persist for relatively short periods, 30 minutes at the most with our membranes, whereas positive anomalous osmotic effects are maintained for an hour or even longer. With the high concentration necessary for negative osmosis, the concentration gradient across the membrane is initially very steep, as diffusion proceeds, the electrolyte concentration in all parts of the membrane is increased and soon becomes high enough to depress electrokinetic effects. At such time, spontaneous electroosmosis (that is, negative osmosis) ceases and true osmotic effects are then observed, the direction of fluid flow being reversed.

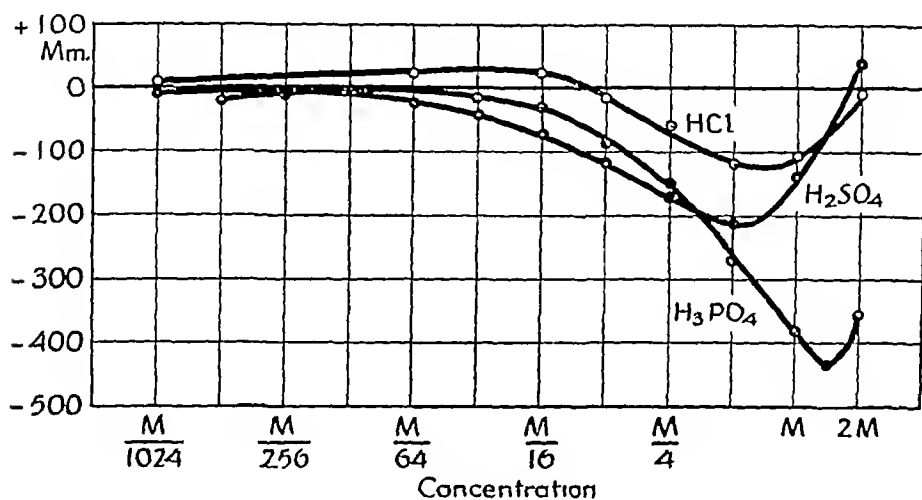


FIG 3

To further extend the analogy between the porous protamine and the porous oxidized membranes, comparative electroosmosis experiments were performed with protamine, oxidized, and unoxidized membranes.

Several collodion membranes were prepared from Baker, U.S.P., collodion according to the method described above. One third of this group was used as such, a second third was oxidized for 3 hours with molar sodium hypobromite, and the remaining third was impregnated with isoelectric protamine. Following a thorough washing, all of these membranes were subjected to electroosmosis experiments for which a current of 10 milliamperes was passed through the membrane, the latter being attached to a rubber stopper holding a graduated manometer tube of 1 cm diameter. The electrodes consisted of a platinum wire spiral inside the membrane and a platinum wire cage outside.<sup>2</sup> In each experiment, a current of 20 milliamperes was applied for 10 minutes. Then the current was then reduced to 10 milliamperes and after some time the volume transported in 10 minutes was recorded. The polarization before the actual measurements was necessary (especially with the dilute solutions) in order to have a constant condition of polarization during the actual measuring.

period. The electrolytic solutions used were 0.1, 0.01, and 0.001 M potassium chloride. The results of these experiments with a membrane representative of each group are given in Table I, the volume changes being expressed as milliliters per hour.

The similarities in magnitude of the electroosmotic transports with protamine and oxidized membranes are obvious, those of the unoxidized membranes fall considerably below this level.<sup>21</sup>

A few words concerning the physical properties of the porous protamine-collodion membranes may be added here. The pore space of our membranes is about 80 per cent of the actual membrane volume, the concentration potentials obtained with various electrolytes across such membranes are only a few millivolts different from those obtained on free diffusion. This situation is quite analogous to the observations with the oxidized porous collodion membranes.<sup>2</sup>

TABLE I

*Electroosmosis through Protamine-Collodion Oxidized and Unoxidized Collodion Membranes (Current 10 M Amps., Membrane Area about 100 Cm.<sup>2</sup>)*

Concentration of KCl solution	Electroosmotic flow per hr		
	Protamine-collodion membrane	Oxidized collodion membrane	Unoxidized collodion membrane
moles per liter	ml.	ml.	ml.
0.001	-4.38	+4.64	+2.84
0.01	-1.68	+1.80	+0.36
0.1	-0.08	+0.21	+0.00

\* The plus sign indicates transport towards the cathode, a minus sign transport towards the anode.

The ohmic resistance of such membranes (thickness of 0.03 to 0.04 mm.) when immersed in 0.05 M potassium chloride solution, is very low, about 1 ohm—on the average—per 100 cm.<sup>2</sup> of membrane, the ohmic resistance is not significantly different from the original membranes which are not coated with protamine.

The rate of filtration of water or electrolyte solutions through such membranes seems to be somewhat lower than that observed with the ordinary collodion membranes. For example, the filtration rates through three of our typical membranes previous to the adsorption of protamine were 1.83, 2.35, and 1.55 ml per hour under an average hydrostatic head of 15 cm. of water, the filtering area being roughly 100 cm.<sup>2</sup> After the adsorption of the protamine, these values were 1.61, 1.77, and 1.18 ml per hour. To minimize disturbances which could be caused by electrical forces, molar potassium chloride solution

<sup>21</sup> Electroosmotic transport values given previously (Sollner, K. Abrams I., and Carr C W *J Gen Physiol* 1941, 25, 16) were erroneously expressed as transport values per 20 minutes; they are actually transport rates per hour.



was used in these filtration experiments. This observation agrees qualitatively with the observation of Hitchcock<sup>23</sup> that the filtration rate of collodion membranes after the adsorption of gelatin was considerably reduced, the stronger effect with gelatin being due most likely to its greater molecular size.

We may add that membranes exposed to an isoelectric protamine solution assume a slightly yellowish tinge. This effect is probably not due to the protamine, but rather to the alkalinity of the solution. Membranes immersed in a slightly alkaline solution without protamine for relatively long periods also assume a yellow coloration. Whatever the exact nature of this effect may be, it seems to be of no significance for the structure of the membrane.

#### IV

As was mentioned previously, stable electropositive membranes which correspond in their properties to the activated dried collodion membrane are not described in the literature.

Protamine and its salts are practically insoluble in ether-alcohol. Therefore, dried positive membranes cannot be prepared from collodion solutions containing protamine in the dissolved state.

The adsorption of protamine on dried collodion membranes also does not yield satisfactory results, it was found that the negative character of the membranes is only slightly reduced, in no case was the electrokinetic charge reversed. This is easily understood if we consider the fact that the pores of dried collodion membranes are too small to allow the entry of molecules as large as protamine. Thus, the protamine is excluded from the very spots which determine the electrochemical characteristics of the membrane.

A successful approach to the problem consists of adsorbing protamine on porous collodion membranes followed by drying. The technique is quite simple. Porous protamine membranes are prepared as described in a preceding section and dried in air for at least 6 hours. After this time, they assume a shrunken, irregularly wrinkled appearance and are ready for use.

Their most characteristic property is, of course, their electromotive behavior. The characteristic concentration potential— $\text{KCl } 0.1 \text{ M} / \text{KCl } 0.01 \text{ M}$ —closely approaches the thermodynamically possible maximum. Some typical values are given in Table II.

In another series of six such membranes, the concentration potentials at the end of 3 months were found to vary between  $-47$  and  $-52$  mv. Such membranes offer relatively little resistance to the passage of current just as do the dried collodion membranes which are activated by oxidation or prepared from activated collodion.

<sup>23</sup> Hitchcock, D., *J. Gen. Physiol.*, 1925, 8, 61.

TABLE II

*Characteristic Concentration Potentials across Dried Protamine Collodion Membranes +KCl  
0.1M/KCl 0.01M—*

Membrane	Concentration potential after storage in water			
	1 day	1 wk.	2 wks.	3 wks.
	mv.	mv.	mv.	mv.
a	-52.2	-47.5	-49.5	-48.7
b	-50.7	-49.2	-48.0	-48.2
c	-51.4	-51.0	-50.5	-49.5
d	-50.1	-48.7	-48.9	-49.0
e	-48.6	-49.7	-48.0	-46.2
f	-51.0	-50.3	-49.4	-47.6

Thus, the experimental evidence indicates that the dried protamine-collodion membrane can be considered as an electropositive analogue of the dried activated collodion membrane—just as the porous protamine-collodion membrane is an electropositive analogue of the porous activated collodion membrane.

#### SUMMARY

1 Strongly electropositive porous membranes were prepared by the adsorption of protamine (salmune) on porous collodion membranes. These membranes retain their electrochemical characteristics for at least 12 months without change.

2 They are distinctly electropositive between pH 1 and 10, the range of most pronounced electropositive behavior occurring in solutions between pH 3 and pH 8. The filtration rates and ohmic resistance of these membranes do not differ significantly from similar uncoated membranes.

3 The porous protamine-collodion membranes show very pronounced positive anomalous osmosis, the observed effects with proper electrolytes being similar to those obtained with oxidized collodion membranes. They also show very conspicuous negative osmosis with strong acids.

4 Protamine-collodion membranes which correspond in their properties to the activated dried collodion membranes were prepared by the adsorption of protamine on porous collodion membranes followed by drying in air. The concentration potentials across such dried protamine-collodion membranes closely approach the thermodynamically possible maximum.



# SOME THERMOANALYTIC STUDIES OF ORGAN AND WHOLE ANIMAL RESPIRATION

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## INTRODUCTION

These studies are an attempt to obtain comparative information about the thermal behavior of the different organs in an animal body. From these data, and from similar data about the thermal behavior of the whole animal, it is possible to draw certain inferences regarding the conditions which govern the oxygen consumption of tissues. Such inferences are the chief interest of the present paper. More extensive investigations of this sort, however, might also greatly clarify problems of the individual metabolism in tissues. This last question we shall touch upon briefly.

## Technical Details

All the present experiments were carried out on tissues of the winter variety of *Rana pipiens*. The organs used were liver, kidney, and external and internal oblique muscles. Tissue slices were made free-hand with an ordinary razor blade (1), after some practice these were consistently turned out with a thickness of less than 0.2 mm. These slices were suspended in unbuffered Locke's solution.<sup>1</sup> It was established by preliminary experiments (see below) that with the solution and amounts of tissue used (< 50 mg), the increasing acidity of the system was a negligible factor for a period of at least 2 hours. (Readings were subsequently taken for a period of 1 hour only.) The respirometers used were simple manometric systems (American Instrument Co.). Temperature control good to 0.1°C. was obtained. "Dry weight" was determined as the constant weight attained after drying in an oven at 86°C.

*The Results of Preliminary Experiments*—It is, of course, an essential assumption in the measurements of tissue respiratory rates that the determined rate be independent of the duration of the run or of the excision time. This is not the case if the tissue has a high cytolytic rate (rapid negative acceleration of the oxygen consumption). In order to investigate the cytolytic behavior of these tissues, the curve of  $Q_{O_2}$  vs. time was obtained in a large number of experiments lasting an average of 8 hours. In each case it was found that the tissues exhibited a constant  $Q_{O_2}$ , i.e.  $dQ/dt = 0$ , for at least 2 hours after the

NaCl	7.0 gm li <sup>-1</sup>
CaCl <sub>2</sub>	0.316
KCl	0.189
Glucose	1.000

beginning of the experiment or 2 hours and 25 minutes after excision. On the basis of this fact it was decided to take total oxygen consumption during

TABLE I  
*QO<sub>2</sub> Values for Various Organs at Different Temperatures*

Liver	Kidney	Muscle
25 6°		
1 86 (10)*	1 93 (5)	0 324 (9)
30 0°		
0 782 (6)	2 72 (5)	0 282 (5)
20 0°		
1 18 (7)	1 58 (9)	0 46 (9)
15 0°		
0 606 (5)	1 385 (5)	0 389 (5)
10 0°		
0 467 (5)	1 01 (5)	0 279 (5)
5 0°		
0 310 (5)	0 816 (5)	0 322 (5)
0 0°		
0 29 (5)	0 58 (5)	0 27 (5)

\* Numbers in parentheses signify No. of determinations used in calculating the mean given

the 1st hour as the value of the  $Q_{O_2}$ . At all temperatures the second differences of the pressures ( $\Delta^2 p$ ) were always checked to establish the absence of a negative acceleration. In no case was this detected during the time of the run. This is, of course, in striking contrast to the behavior of mammalian tissues

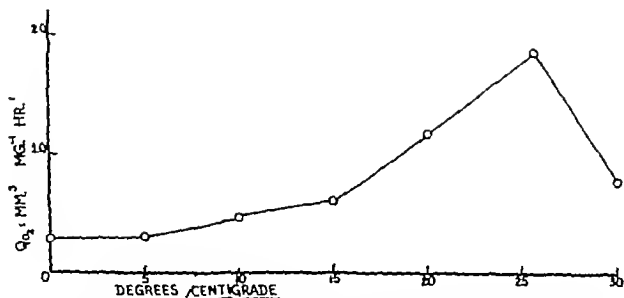


FIG. 1 Oxygen consumption in  $\text{mm}^3 \text{hr}^{-1} \text{mg}^{-1}$ , for liver slices (*Rana pipiens*) between 0 and 30°C. The measures were made at approximately 5° intervals.

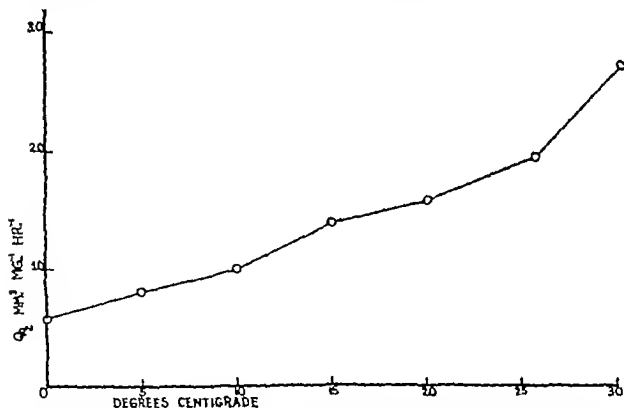


FIG. 2 Oxygen consumption in  $\text{mm}^3 \text{hr}^{-1} \text{mg}^{-1}$ , for kidney slices (*Rana pipiens*) between 0 and 30°C. The measures were made at approximately 5° intervals.

The initial constancy of the  $Q_{O_2}$  has the corollary that the acidity of the tissue did not undergo a non physiological displacement, for such a displacement would necessarily bend the time curve away from linearity. Thus the absence of a buffer was justified.

It might be remarked in passing that much of interest could probably be

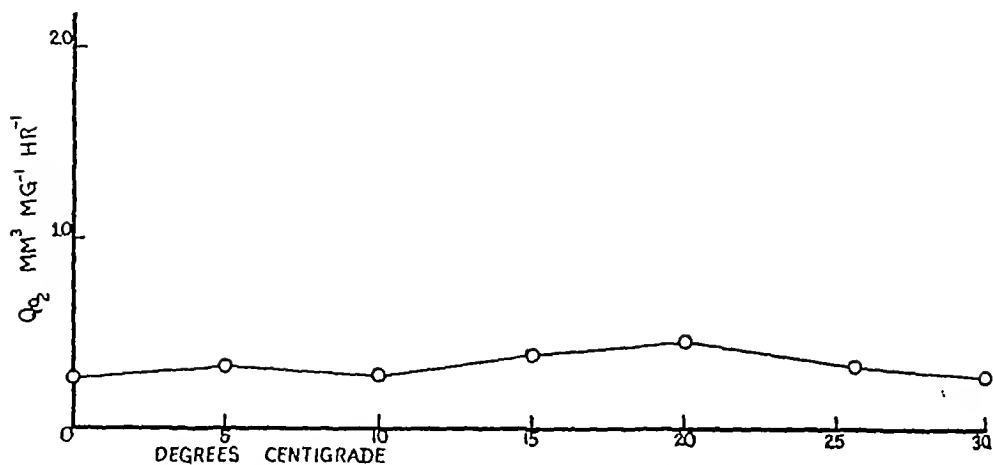


FIG 3 Oxygen consumption in  $\text{mm}^3 \text{hr}^{-1} \text{mg}^{-1}$ , for slices of the oblique abdominal muscles (*Rana pipiens*) between 0 and 30°C. The measures were made at approximately 5° intervals

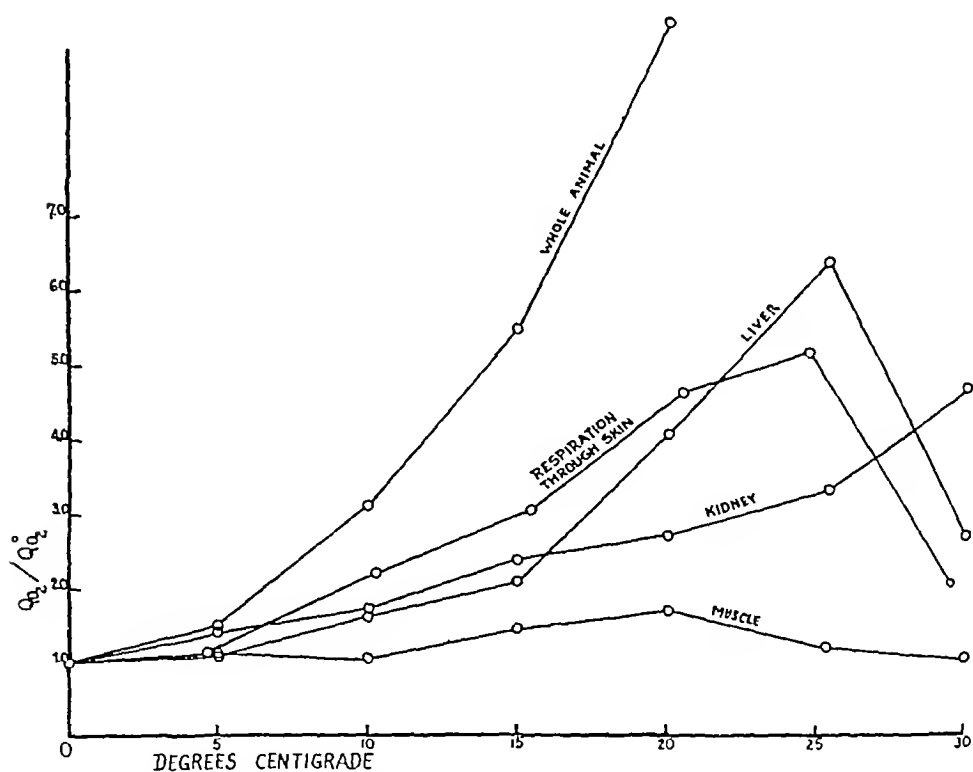


FIG 4 Oxygen consumption of organs and animal referred to oxygen consumption for the same organ or animal at 0°C (*Rana pipiens*). The ratios are given at approximately 5° intervals

gained by performing a rational analysis of the time curves. The constants of such curves would serve as comparative indices for the tissues.

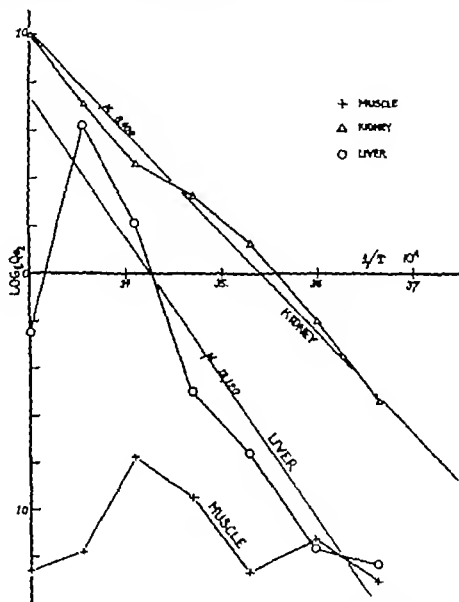


FIG 5 Arrhenius plots of  $\log$  (oxygen consumption) vs the reciprocal of the absolute temperature. Straight lines have been oriented through the points in order to secure approximate values of  $\mu$ .

## RESULTS

The results of all experiments are summarized in Table I, in which entries are made so that for a given tissue (at a given temperature) there are at least five determinations on different animals. The means of readings are then used variously in a series of graphs (1)  $Q_{O_2}$  vs Centigrade temperature (2) A comparison of the various tissues and of the whole animal, using the dependent variable,  $Q/Q^\circ$ , where  $Q^\circ$  is the oxygen consumption at  $0^\circ\text{C}$ . (3) Plots of  $\log Q_{O_2}$  vs  $1/T$  for the various tissues and for the whole animal (4)  $Q_{O_2}$  vs time



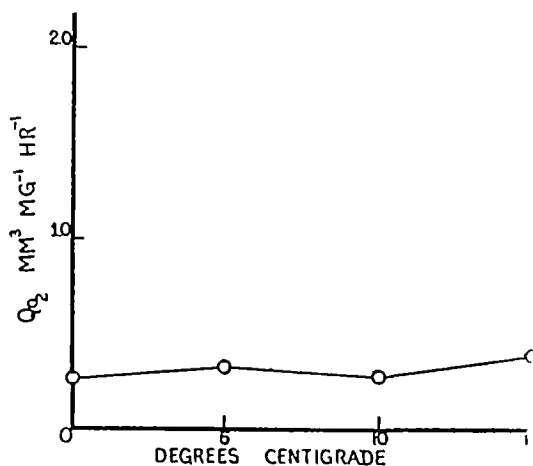


FIG 3 Oxygen consumption in mm<sup>3</sup> hr<sup>-1</sup> muscles (*Rana pipiens*) between 0 and approximately 5° intervals

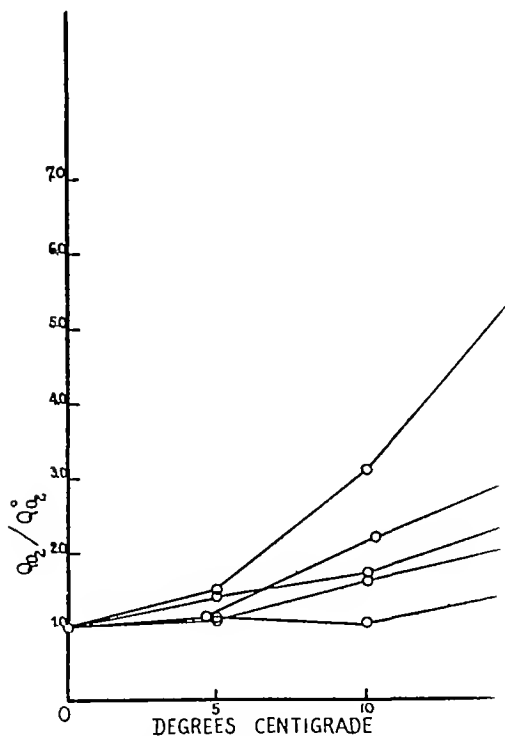


FIG 4 Oxygen consumption of organs for the same organ or animal at 0°C approximately 5° intervals

of various organs might have  
The coincidence or non-  
significance than the coinci-  
which we wish to undertake  
analysis to the problem  
by the availability of the  
which uses up the gas? A  
is assumed that the uptake

$\left\{ \begin{array}{l} O_2 \text{ metabo-} \\ \text{ling in} \\ \text{the tissue} \end{array} \right\}$

all process (whole animal  
tissue respiration) If these  
then it follows that the  $\mu$   
value of the first step, and  
limiting process or "master  
process" is large, then, according  
This view is not shared  
the original condi-

the process,  $\xrightarrow{f} \xrightarrow{g}$

for the whole animal  
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for the whole animal (a difference of about 8,000 cal.), but it is practically equal to that for whole animal respiration through the skin. From this we can very tentatively conclude that whenever lung respiration predominates, the oxygen consumption of the liver is limited by the availability of gas, but that whenever skin respiration predominates no master reaction can be said to exist. It is curious to note in this connection that the  $Q/Q^{\circ}$  curves for liver and for respiration through the skin are strikingly similar.

Finally, it might be remarked in passing that the form of the curve in the Arrhenius plot may turn out to be of great significance even when curvilinear (as is the case for liver in these experiments). If we accept the statistical form of the velocity constant (see, for instance, 10), it follows that by the operation of taking logarithms this function will always be split up into two terms, one of which will contain the energy of activation and  $1/T$ . The temperature dependence of the other term is what may decide departures of the plot from linearity.

The counsel of Professor S. F. Cook and of Mr. F. Kreutzer in connection with this work is most gratefully acknowledged.

#### SUMMARY

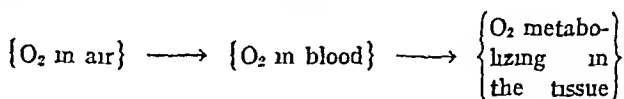
Data on the respiratory rates of frog liver, kidney, and striated muscle were obtained at various temperatures by the Warburg method. Fundamental differences in the curves of  $Q_{O_2}$  vs.  $T$  exist among the tissues and between the tissues and the whole animal. The Arrhenius plots of these curves show that at least for some tissues the availability of oxygen at the tissue is limited by the diffusion of gas through the skin and lungs, governs the  $Q_{O_2}$  of the tissue. Inferences are drawn regarding the comparative metabolism of the tissues and the fallacy of using whole animal  $Q_{O_2}$  alone as a metabolic index.

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tained It is conceivable that the efficiencies for various organs might have optima at approximately equal temperatures The coincidence or non-coincidence of the efficiencies is of course of more significance than the coincidence or non-coincidence of respiratory maxima

*III Arrhenius Plots*—The final consideration which we wish to undertake here is the possibility of applying Crozier's thermal analysis to the problem is the oxygen consumption of a given tissue limited by the availability of the gas or by the metabolic machinery of the tissue which uses up the gas? A theoretical answer to this question can be given if it is assumed that the uptake of oxygen occurs according to the scheme



Let us suppose that we know the  $\mu$  value of the over-all process (whole animal respiration) and also that of the second step (tissue respiration) If these values differ by the requisite Burton amount (3), then it follows that the  $\mu$  value of the over-all process was actually the  $\mu$  value of the first step, and therefore that the first step (availability) is the limiting process or "master reaction" If the difference in  $\mu$  values is insufficiently large, then, according to Burton, nothing can be said regarding mastery This view is not shared by the author,<sup>3</sup> and here we should like to retain tentatively the original condition that if  $\mu_i > \mu_j$ , then  $i$  is the master reaction of the process,  $\xrightarrow{i} \xrightarrow{j}$

To investigate the question we must have the  $\mu$  values for the whole animal (over-all process) and for the chief respiring tissues of the animal For the whole animal the  $\mu$  values are, according to Crozier's (4,5) calculations, 24,000 cal, 21,000 cal, and 11,000 cal, respectively, for Vernon's (6), Krogh's (7,8), and Putter's (9) (respiration through the skin) experiments With respect to the tissues it will be seen that the plots are of various types—a matter which we shall comment on below Kidney  $\text{Q}_{\text{O}_2}$  gives a  $\mu$  value of 8,400 cal Liver does not give a good rectilinear plot (in fact, the points fall on a rather regular curve), but a rough estimate of the characteristic is 12,000 cal The muscle plot is highly irregular, but it is safe to say that the greatest possible value (computed from the maximum slope) is less than 5,000 cal

Using these data in the theory, we are led to the deduction that both for kidney and for muscle the site for the master reaction in their metabolism is *between* the organ and the external air In the frog the site would be at the lungs and possibly on the skin, and the limiting process would be the diffusion of oxygen through these membranes

The case of liver is not so clear cut Its  $\mu$  value is considerably below those

<sup>3</sup> This matter is considered in a paper now in preparation

for the whole animal (a difference of about 8,000 cal), but it is practically equal to that for whole animal respiration through the skin. From this we can very tentatively conclude that whenever lung respiration predominates, the oxygen consumption of the liver is limited by the availability of gas, but that whenever skin respiration predominates no master reaction can be said to exist. It is curious to note in this connection that the  $Q/Q^{\circ}$  curves for liver and for respiration through the skin are strikingly similar.

Finally, it might be remarked in passing that the form of the curve in the Arrhenius plot may turn out to be of great significance even when curvilinear (as is the case for liver in these experiments). If we accept the statistical form of the velocity constant (see for instance, 10), it follows that by the operation of taking logarithms this function will always be split up into two terms, one of which will contain the energy of activation and  $1/T$ . The temperature dependence of the other term is what may decide departures of the plot from linearity.

The counsel of Professor S. F. Cook and of Mr. F. Kreutzer in connection with this work is most gratefully acknowledged.

#### SUMMARY

Data on the respiratory rates of frog liver, kidney, and striated muscle were obtained at various temperatures by the Warburg method. Fundamental differences in the curves of  $Q_{O_2}$  vs  $T$  exist among the tissues and between the tissues and the whole animal. The Arrhenius plots of these curves show that at least for some tissues the availability of oxygen at the tissue, as limited by the diffusion of gas through the skin and lungs, governs the  $Q_{O_2}$  of the tissue. Inferences are drawn regarding the comparative metabolism of the tissues and the fallacy of using whole animal  $Q_{O_2}$  alone as a metabolic index.

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# STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

## VI INHIBITOR STUDIES WITH SUCCINIC DEHYDROGENASE\*

By V. R. POTTER AND K. P. DOBOIS

(From the McArdle Memorial Laboratory, University of Wisconsin Medical School, Madison)

(Received for publication, November 12, 1942)

In 1938, Hopkins and coworkers (1, 2) showed that succinic dehydrogenase could be inactivated by oxidized glutathione (GSSG) and could be reactivated by reduced glutathione (GSH). They interpreted these results to mean that the active enzyme requires intact —SH groups and that when these are converted to the —S—S— form of the enzyme, the dehydrogenase is inactivated. Any assumption that the functioning of the enzyme involved an oscillation between the SH and the —S—S— form of the enzyme seemed to be definitely eliminated, however, by the fact that the —S—S— form could not be reduced by succinate. Thus the function of the SH group in succinic dehydrogenase has remained an unsolved problem.

Although many proteins contain SH groups, very little is known about the structural relationship of the SH group to the rest of the molecule. Even in the case of egg albumin, in which the SH groups have received the most careful study, the mechanism by which the SH groups of native egg albumin are shielded from some sulphydryl reagents and not from others remains obscure (3). In the case of succinic dehydrogenase, the presumptive SH group (1, 2) is associated with function, and the reaction of the protein with sulphydryl reagents should be demonstrable on the basis of determinations of the amount of active enzyme remaining. Furthermore, since it is an oxidative reaction, the measurement of oxygen uptake makes possible a continuous measurement of the amount of active enzyme at any given moment. We have established the test conditions for the measurement of the activity of the enzyme (4, 5). The rate of oxygen uptake is a valid measure of dehydrogenase activity in this system since cytochrome *c* and cytochrome *oxidase*, which are needed to complete the reaction with oxygen, are present in excess. The activity of the enzyme is so great under the proper conditions that the extraneous matter present in the enzyme preparation does not interfere with the study of the reaction.

At present, inhibitor studies appear to constitute the only available means of establishing the presence of SH groups in succinic dehydrogenase and of determining their rôle in the function of the enzyme.

\* This work was supported by the Jonathan Bowman Fund for Cancer Research.



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In the work to follow, the nature of the SH groups in succinic dehydrogenase has been studied by testing the inhibitory action of some 38 different chemical compounds over a wide range of concentrations and in the presence and absence of the substrate. In addition, malonate, which is itself an inhibitor, was shown to decrease the action of other inhibitors. On the basis of the inhibitor experiments, it has been possible to approximate the structure of the succinate-activating center of the dehydrogenase and to provide an explanation for the earlier results of Hopkins, thus reopening the possibility that the SH group may be involved in hydrogen transport in this enzyme.

#### EXPERIMENTAL

*Enzyme System*—Studies in this laboratory (4, 5) have led to the development of an enzyme preparation in which the components of the succinoxidase system are believed to approximate their native functional activity more closely than in systems hitherto studied. Rat liver homogenates were freshly prepared and kept in ice water until pipetted. The reaction mixtures contained 0.8 ml. of 0.1 M sodium phosphate pH 7.4, 0.4 ml. of  $10^{-4}$  M cytochrome *c*, 0.3 ml. of  $4 \times 10^{-3}$  M  $\text{CaCl}_2$ , 0.3 ml. of  $4 \times 10^{-3}$  M  $\text{AlCl}_3$ , 0.3 ml. of 0.5 M recrystallized sodium succinate pH 7.4, 0.1 ml. of 10 per cent liver homogenate in 0.033 M sodium phosphate pH 7.4, plus water and inhibitor solution to give a final volume of 3.0 ml. About once a week the calcium, aluminum, and cytochrome were made up into one solution of which 1 ml. was pipetted into each flask. All solutions were stored in the cold and kept in ice water during the pipetting. The rate of oxygen uptake was measured in a conventional Warburg apparatus at 38°C. The need for the various components has been previously demonstrated (4, 5). The enzyme system as set up will not oxidize succinate beyond the fumarate stage.

*Inhibitor Experiments*—During the course of this work, a wide variety of compounds were tested for toxicity to the succinoxidase system. The results are summarized as briefly as possible in Table I. Although the concentration of inhibitor was varied over a wide range, only one concentration is reported. In most cases, the presence of succinate decreased the action of the inhibitors, therefore, the enzyme was incubated in the absence of its substrate, and the activity was measured by delaying the succinate addition. In these cases, less inhibitor was required for a given amount of inhibition than in the case of experiments in which succinate was present at the beginning. In the delayed succinate experiments, the action of the inhibitor was usually complete by the time the substrate was added, while in those cases where succinate was present from the beginning, the per cent inhibition usually increased with time. In the latter experiments, it is thus necessary to specify the time at which the per cent inhibition was computed, and in Table I the percent inhibition is stated for the second and fourth 10 minute intervals. Although most of the inhibitors would give 100 per cent inhibition if added in sufficient quantity, an attempt was made to report those concentrations which gave 50 to 80 per cent

TABLE I

## Inhibition of Succinoxidase

Test system described in text. Inhibition calculated from  $QO_2$  values with controls in each run.

No.	Compound	Substrate added at 20 min.		Substrate added at the beginning		
		Inhibitor concentration	Inhibition	Inhibitor concentration	Inhibition 10-20 min.	Inhibition 30-40 min.
Quinones and related compounds						
1	Quinone	$\mu/100\ 000$	65	$\mu/30\ 000$	50	68
2	1 4 Naphthoquinone	$\mu/100\ 000$	43	$\mu/30\ 000$	21	30
3	2 Methyl naphthoquinone	$\mu/10\ 000$	53	$\mu/1\ 000$	44	43
4	9 10 Anthraquinone	Saturated	0	Saturated	0	0
5	Hydroquinone	$\mu/100\ 000$	65	$\mu/30\ 000$	11	35
6	Catechol	$\mu/100\ 000$	35	$\mu/30\ 000$	3	5
7	Resorcinol	$\mu/10\ 000$	0	$\mu/1\ 000$	0	0
Azo compounds and diamines						
8	p-Phenylenediamine	$\mu/100\ 000$	65	$\mu/30\ 000$	26	54
9	p-Aminophenol	$\mu/30\ 000$	43	$\mu/100\ 000$	17	46
10	o-Phenylenediamine	$\mu/30\ 000$	37	$\mu/1\ 000$	7	7
11	N Methyl-p-phenylenediamine	$\mu/100\ 000$	65	$\mu/30\ 000$	43	72
12	p-Aminodimethylaniline	$\mu/100\ 000$	71	$\mu/30\ 000$	45	77
13	2 Methyl N <sup>N</sup> dimethyl 1 4 phenylene-diamine (meta)*	$\mu/100\ 000$	72	$\mu/30\ 000$	15	26
14	2 Methyl N <sup>N</sup> dimethyl 1 4 phenylenediamine (ortho)*	$\mu/100\ 000$	68	$\mu/30\ 000$	0	0
15	p-Aminoacetanilide	$\mu/1\ 000$	4	$\mu/100$	50	53
16	Sulfanilamide	$\mu/100$	0	$\mu/100$	0	0
17	p-Nitrosodimethylaniline	$\mu/2\ 000$	4	$\mu/2\ 000$	22	18
18	p-Aminoazobenzene	$\mu/2\ 000$	43	$\mu/2\ 000$	36	35
19	p-Dimethylaminoazobenzene†	$\mu/200$	55	$\mu/200$	25	24
20	Methyl orange	$\mu/1\ 000$	33	$\mu/200$	42	48
21	Methylene blue	$\mu/3\ 000$	63	$\mu/3\ 000$	20	20
Sulphydryl reagents						
22	Iodine	$\mu/3\ 000$	52	$\mu/3\ 000$	4	4
23	Iodoacetate	$\mu/1\ 000$	52	$\mu/100$	17	31
24	Iodoacetamide	$\mu/1\ 000$	90	$\mu/100$	68	83
25	Maleic acid	$\mu/25$	17			
26	p-Chloro-mercuro-benzoic acid	$\mu/100\ 000$	30	$\mu/30\ 000$	63	70
27	Ferricyanide	$\mu/1\ 000$	89	$\mu/1\ 000$	32	58
Metal cations						
28	Copper <sup>++</sup>	$\mu/100\ 000$	64	$\mu/30\ 000$	67	83
29	Zinc <sup>++</sup>	$\mu/100\ 000$	30	$\mu/30\ 000$	56	63
30	Iron <sup>+++</sup>	$\mu/3\ 000$	29	$\mu/1\ 000$	32	58
Toxic anions						
31	Selenite <sup>---</sup>	$\mu/100\ 000$	46	$\mu/30\ 000$	11	20
32	Arsenite <sup>---</sup>	$\mu/10\ 000$	25	$\mu/1\ 000$	16	42

Obtained through the kindness of Dr. C. J. Kenner Memorial Hospital New York.

† Dissolved in corn oil and homogenized with the tissue. Corn oil or dye non toxic alone.

inhibition for the majority of the compounds and to report the inhibition obtained at this same concentration with compounds which were less toxic

Although it is not possible to discuss each inhibitor at length and to give all the reasons which led to their selection, it may be stated that each compound serves as a control for one or more other compounds in the series, and all of the compounds studied are reported for the sake of completeness. Of the 38 compounds listed, certain key compounds enable one to deduce the nature of the active center in succinic dehydrogenase

It is desirable at the outset to emphasize the oxidative capacity of the enzyme system. Many of the compounds used are in the reduced form (*p*-phenylenediamine, *p*-aminophenol, hydroquinone, etc.), but in the presence of cytochrome *c* they are rapidly oxidized. It seems certain that the toxicity of these compounds arises from their properties in the oxidized state, as Potter (6) demonstrated in studies with the urease system. This conclusion is supported by the results with the succinoxidase system

The key compound of the series is *quinone* (*p*-benzoquinone) (No. 1 in Table I), and 21 of the 38 compounds studied are related to this substance. Of the 21 compounds, those which are toxic either contain the quinonoid structure or are converted to a quinonoid structure in this system. It seems likely that any quinonoid compound will inhibit succinic dehydrogenase but that additional factors such as solubility, molecular size, and configuration lower the toxicity as the compound deviates from the properties of quinone. Thus it will be seen that quinone is the most toxic compound in the quinonoid series, although many of the diamines are equally toxic (the difference between 65 per cent and 72 per cent inhibition is not considered significant). Naphthoquinone (No. 2) is nearly as toxic as quinone, but the introduction of a methyl group to give *2-methyl naphthoquinone* (artificial vitamin K) (No. 3) decreases the toxicity almost tenfold<sup>1</sup>. It has been shown (7) that quinone will combine with the SH group, and Fieser (8) has carried out a number of chemical studies demonstrating the same reaction with 2-methyl naphthoquinone. Although the 2-methyl naphthoquinone was also shown to react with certain alcohols and amino acids, it is interesting to note that Fieser concluded, "it is likely that methylnaphthoquinone can combine with proteins most readily by utilization of the sulfhydryl groups rather than the  $\epsilon$ -amino groups". In the case of the next compound, *9,10 anthraquinone* (No. 4), no toxicity whatsoever could be observed (Table I), and it is obvious that the reaction postulated for quinone could not occur with this compound

<sup>1</sup> The toxicity observed clinically with high doses of this compound (8) may be due to interaction with succinic dehydrogenase or a similar enzyme. The tenfold decrease in toxicity resulting from the introduction of one methyl group provides a explanation for the absence of clinical toxicity with *natural* vitamin K since it contains a long side-chain at the 3 position in addition to the 2-methyl group, and reaction with a sulfhydryl group is manifestly impossible

Turning now to the various quinols, it is seen that *hydroquinone* (quinol) (No 5) is just as toxic as quinone under strongly oxidizing conditions. When oxidation is less because of succinate, the compound is less toxic than quinone. This result, plus the data on quinone, proves that the action of succinate is twofold: it protects the enzyme against the inhibitors directly, and it slows the conversion of reduced compound to the toxic oxidized form. Catechol (No 6), which is oxidized to the orthoquinone, further demonstrates this point. Resorcinol (No 7), which is not autooxidizable and does not reduce cytochrome *c*, is not oxidized in this system. Thus it cannot be converted to the quinonoid structure and is completely non toxic.

A number of compounds in which the OH groups of the quinols are replaced by amino groups have as the key compound *p*-phenylenediamine (No 8), this is analogous to hydroquinone. A mixed compound is *p*-aminophenol (No 9) in which only one of the OH groups is replaced by  $\text{—NH}_2$ . The ortho structure is represented by *o*-phenylenediamine (No 10). Methyl groups can be substituted for hydrogen on the ring or in the amino groups (compounds 11 to 14) without loss in toxicity. Compound 14 is not autooxidizable and gives no inhibition when its oxidation by cytochrome is hindered by succinate. All of the toxic amino compounds are oxidized to the quinonoid structure. Various deviations which decrease the ease of conversion to the quinonoid structure result in great decreases in toxicity. This is most striking in the case of *p*-aminoacetanilide (No 15) which is essentially non toxic and is not oxidized to the quinonoid structure in this system. It may be contrasted with *N*-methyl-*p*-phenylenediamine (No 11) which has a very similar structure but is very toxic and is readily oxidized to the quinonoid dimer. Further illustration of this point is sulfanilamide (No 16) and *p*-nitrosodimethylaniline (No 17) which are not oxidized and the azotized compounds *p*-aminoazobenzene (No 18) and *p*-dimethylaminoazobenzene (No 19). The latter compound is quite insoluble in water and, when sulfonated, forms methyl orange (No 20) which is water soluble and much more toxic than its parent compound though it has less than 1 per cent of the toxicity of quinone. It forms the quinonoid structure but is probably less toxic because of the size of the molecule. The same is probably true for methylene blue (No 21) which is toxic and possesses the quinonoid structure. Cedrangolo and Adler (9) have shown that methylene blue will oxidize the SH of cysteine and glutathione and that it will inhibit triosephosphate dehydrogenase. They suggested that the mechanism of the inhibition was interaction with the SH of the enzyme. A number of other oxidation reduction dyes have been shown (10) to be toxic for the succinic system, with toxicity being a function of the potential of the dye. Phenothiazone (11) and pyocyanine (12) also inhibit succinic dehydrogenase. All of the toxic compounds mentioned thus far possess the quinonoid structure. Although they might conceivably react with some group other than the SH group of the enzyme, the evidence seems to point to the SH group.

However, it seems desirable to consider the effect of other compounds which are toxic but which do not possess the quinonoid structure. A number of reagents have been used to titrate the SH groups of proteins such as egg albumin or to inhibit enzymes on the basis of interaction with SH. The literature on most of these compounds has been reviewed by Hellerman (13, 14). The compounds include iodine (No 22), iodoacetic acid (No 23), iodoacetamide (No 24), maleic acid (No 25), *p*-chloro-mercuro-benzoic acid (No 26), and ferricyanide (No 27). Several of these compounds have been shown to react *stoichiometrically* with the SH groups of denatured egg albumin (3) but with varying degrees of completeness in the case of the native egg albumin. It seems reasonable to conclude that they react with the SH groups of succinic dehydrogenase in a similar manner and that the latter are simply less accessible to these compounds than to quinone.

Many metallic ions are known to react with sulfhydryl groups (2, 13, 14). Experiments with copper (No 28), zinc (No 29), and iron (No 30) are included in Table I. The ferric ion is much less toxic than zinc and copper. The latter is stoichiometrically equivalent to quinone, and the rate of inactivation is about the same. The ability of copper to react with thiols has been amply demonstrated by Pirie (15) who prepared a number of crystalline copper derivatives of SH compounds. In addition to the heavy metal cations which react with thiols, certain anions will also combine with the SH grouping. These are the selenite ion (No 31) and the arsenite ion (No 32). Bersin (16) reported that thiol compounds will combine with selenite, while Johnson and Voegtlin (17) demonstrated their combination with arsenite. The latter workers prepared a number of crystalline derivatives and postulated that the toxic action of arsenite is due to chemical combination with cellular SH compounds essential to life.

The above 32 compounds represent quinonoid structures, SH reagents, metal cations, and anions. The most toxic compounds are quite similar in their action on the basis of rate of reaction, final inhibition, and effective molarity. There can be little doubt that the common denominator of all these inhibitors is their reaction with the sulfhydryl group of succinic dehydrogenase. The situation is similar to that with egg albumin, concerning which Anson (3) stated, "the SH group, however, is the only protein group known to react with both oxidizing agents and heavy metal compounds."

In the case of the studies with urease (6), it was concluded that the mechanism of reaction between the SH groups and the inhibitors was more likely to be a combination rather than an oxidation of  $\text{EnSH}$  to  $\text{En-S-S-En}$ . The same conclusion is reached in the present instance and, since the line of reasoning is the same, it need not be repeated here. The inactivation by combination with SH probably occurs with all the quinonoid compounds, SH reagents, metal cations, and toxic anions and is represented diagrammatically in

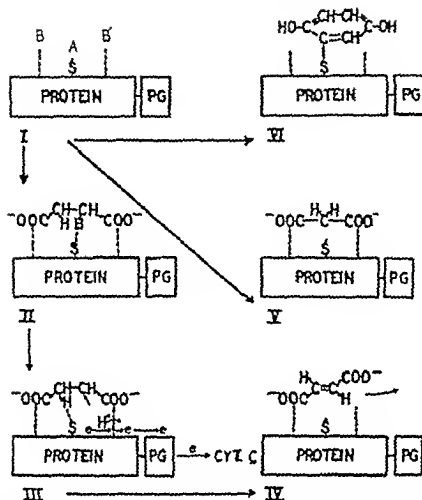


FIG 1 Schematic representation of the structure of succinic dehydrogenase on the basis of *mutually exclusive* inhibitor reactions. The protein is succinic dehydrogenase and PG represents its hypothetical prosthetic group

I = enzyme showing the succinic acid activating center A is a sulfhydryl amino acid e.g. cysteine, in a peptide chain B and B' are the carbonyl affinity points e.g.,  $-\text{NH}-$  groups of adjacent peptide linkages capable of forming hydrogen bonds with the carbonyl oxygens.

II = enzyme-succinic acid complex in first stage of succinic acid oxidation

III = enzyme-succinic acid complex in a possible intermediate stage of succinic acid oxidation

IV = enzyme-fumaric acid complex resulting from the oxidation of succinic acid

V = enzyme-malonic acid complex. Only the carbonyl affinity points are involved Inhibition reversible. Enzyme-sulfhydryl shielded by malonate and thereby protected against sulfhydryl reagents.

VI = enzyme-quinol complex, as an example of inhibition by a sulfhydryl reagent (quinone) Analogous complexes formed with other quinonoid compounds, thiol reagents thiols heavy metals arsenite and selenite.

Fig. 1, stage VI, using quinone as the inhibitor In the case of urease, it was possible to *prevent* but not to *reverse* the inactivation due to quinones by adding cysteine to the reaction mixture This fact gives further support to the

postulated mechanism. However, such experiments are not possible with the present succinic system since, in contrast to the urease system, cysteine is oxidized to a form which inactivates the succinic dehydrogenase as is shown in the next section.

*Inhibition by Sulfhydryl Compounds*—In the original experiments by Hopkins *et al.* (1, 2), it was found that  $R-S-S-R$  would inactivate succinic dehydrogenase and that  $RSH$  would restore the activity. We have tested our succinoxidase system and have found that both cysteine and cystine inactivate the enzyme. Reduced glutathione will cause some inactivation (see Table II). The apparent disagreement with Hopkins *et al.* is only superficial, however, and is easily understood when the respective experimental techniques are considered. Hopkins and his coworkers added large amounts of the sulfhydryl com-

TABLE II  
*Inhibition of Succinoxidase by Sulfhydryl Compounds*

Substrate addition delayed for 30 minutes. Egg albumin "molarity" is the cysteine molarity of the albumin used. Test system as described in text. Activity based on the 10 to 40 minute period after addition of substrate.

Inhibitor molarity	Inhibition				
	Cystine	Cysteine	Reduced glutathione	Egg albumin	
				Native	Denatured
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
M/500		93	18	0	0
M/1,000		69	0	0	0
M/3,000	51*	55*	0	0	0

\* Inhibition was zero when succinate was added originally.

pounds to washed muscle pulp, incubated the mixture, and then washed out the excess of soluble sulfhydryl compound on a Buchner funnel. Thus, the  $SH$  compound was never present when the activity of the enzyme was being tested. In our work, we used considerably less enzyme in much greater dispersion so that in fact it could not be washed on a funnel and recovered. Thus, we add much less sulfhydryl and leave it in the final reaction mixture, where it is converted into the oxidized form. Separate experiments in the absence of the enzyme show that  $RSH$  is oxidized by cytochrome  $c$ , and it seems highly probable that the free sulfhydryl radical  $RS\cdot$  must be formed as an intermediate in such a reaction even though it dimerizes rapidly to  $R-S-S-R$  (14). It seems more likely that the enzyme is inactivated by the formation of  $En-S-S-R$  than by oxidation to  $En-S-S-En$  (in Hopkins' experiments as well as our own). In either case, the reactivation is undoubtedly due to the splitting of the disulfide linkage by  $RSH$  to reconstitute  $En-SH$ . Table II also shows

that native egg albumin, which will not reduce cytochrome *c*, is non toxic at concentrations where cysteine is effective. Denatured egg albumin has free SH groups which rapidly reduce cytochrome *c*, but due to steric hindrance these oxidized SH groups cannot react with the SH of native succinic dehydrogenase as effectively as cysteine. However when the concentration of egg albumin, native or denatured, is raised to higher levels, succinic dehydrogenase can be inhibited. This fact is perhaps analogous to the fact that large quinonoid molecules are much less toxic than quinone but are nevertheless toxic (Table I). The rate of inactivation of the dehydrogenase by the smaller sulphydryl compounds in the presence of cytochrome system is about the same as the inactivation by copper, quinone and all of the other inhibitors which are believed to combine with the SH group of the enzyme. It is generally agreed that inactivation of an enzyme by  $R-S-S-R$  is due to interaction with the sulphydryl of the enzyme (1, 2, 13, 14) and such a conclusion is indicated here. Thus, inhibition of the enzyme by the oxidized sulphydryl compounds appears to proceed by the same mechanism as in the case of the quinonoid compounds, heavy metals, sulphydryl reagents, arsenite and selenite.

*Inhibition by Malonate*—In contrast to all of the previously mentioned inhibitors which are believed to inactivate the succinic dehydrogenase by combining with the SH group of the enzyme, malonic acid appears to inhibit succinic dehydrogenase by an entirely different mechanism. Malonate is a strong inhibitor of the succinic dehydrogenase because it possesses two  $-COOH$  groups and a configuration very similar to that of succinate (18, 19). Previous experiments with minced tissue or slices appear to be complicated by diffusion effects and require up to 0.025 M malonate to block the enzyme completely (20). According to Krebs and Eggleston (20), the inhibition is competitive, *i.e.*, it does not depend upon the absolute concentration of malonate but on the ratio, succinate/malonate. These workers found an inhibition of 50 per cent when the ratio was 9.5/1. The data in Table III confirm their observation that inhibition by malonate is competitive. However when these data are used to calculate the succinate/fumarate ratio at the points of 50 per cent inhibition, one obtains values of 57.2, 51.8, and 50.6 for the three succinate concentrations employed. These ratios represent a close approximation of the ratios between the dissociation constants for the enzyme-succinate and enzyme-malonate complexes and are about 10 times as high as those reported by Krebs and Eggleston. The difference is probably due to the fact that in our preparation the enzyme is operating at maximum activity. The data support the view that malonate and succinate form similar dissociable complexes with succinic dehydrogenase and that the same affinity points are involved. The nature of these complexes is illustrated diagrammatically in Fig. 1, stages II (succinate) and V (malonate). That the affinity points involve the two carboxylic acid groups is indicated by previous work (2, 18, 19). There is no evidence contrary



to the statement by Hopkins *et al* (2) that "malonic acid apparently establishes no special relations with thiol groups"

*Shielding Action of Malonate*—Hopkins *et al* reported that malonate and succinate would prevent the inactivation of succinic dehydrogenase by GSSG. We have confirmed the protection by succinate against the SH inhibitors (Table I) and against cysteine and cystine (Table II) and now present evidence demonstrating protection of the enzyme by malonate against the action of quinone (See Fig 2). Similar results were obtained with *p*-phenylenediamine. The data in Fig 2 also show the difference between the malonate and the quinone inhibition. The results are expressed in terms of the  $Q_{O_2}$ , and the observed rate for each 10 minute period is plotted against time. The  $Q_{O_2}$  value

TABLE III

*Inhibition of Succinoxidase by Malonate Effect of Substrate Concentration*

Test system described in text Succinate and malonate both added prior to enzyme  
 $Q_{O_2}$  calculated on basis of 1st 40 minutes

Substrate concentration	Malonate concentration			
	0	$M/300$	$M/1,000$	$M/3,000$
	$Q_{O_2}$	$Q_{O_2}$	$Q_{O_2}$	$Q_{O_2}$
$M/15$	101	30	61	79
$M/20$	102	24	49	74
$M/30$	101	13	36	63

thus gives the amount of active enzyme present at any given moment. A detailed examination of Fig 2 shows the following

(a) Quinone inhibits progressively as shown in curve 2, with almost no inhibition originally and approaching complete inhibition after 60 minutes. When the enzyme is not protected by succinate, the rate of inactivation is at least twice as rapid, as shown by curve 4. These results show that succinate protects the enzyme against quinone and that, when the quinone combines with enzyme, the combination remains inactive. Apparently quinone can react only with enzyme molecules which are not combined with succinate.

(b) Malonate exerts its action at once, and the per cent inhibition remains constant (curve 3), the inhibition is no greater when the malonate is incubated with the enzyme in the absence of succinate (curve 5). These results, together with the data in Table II, show that malonate forms a dissociable complex with the enzyme which is comparable to the true enzyme-substrate complex and, furthermore, that the same affinity points are involved for succinate and malonate, that is, malonate does not damage the enzyme but merely competes with succinate for the succinate affinity points.

(c) Malonate can prevent quinone from acting on succinic dehydrogenase

whether succinate is added at once (curve 6) or after an incubation period (curve 7). Since the dissociation constant for the enzyme-malonate complex is only about one-fiftieth that of the enzyme-succinate complex (see above), the failure of quinone to inhibit in the presence of malonate is probably due to the fact that there are virtually no uncombined enzyme molecules available

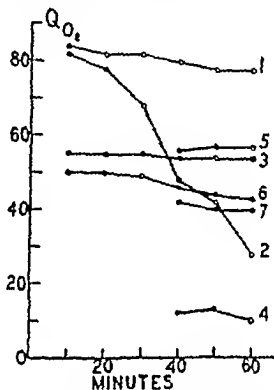


FIG. 2. Shielding of succinic dehydrogenase by malonate against quinone. Rate of inactivation of succinoxidase system (i.e., rate of change in  $QO_2$ ) as effected by malonate and quinone, separately and together with original and delayed succinate additions. See Fig. 1, stages I, II, V and VI for graphic interpretation of results. Test system as described in text. Final inhibitor concentrations: malonate,  $M/3,000$ , quinone,  $M/50,000$ . 1 control—neither inhibitor added, succinate originally present. (Loss in enzyme activity is negligible when succinate addition is delayed 35 minutes.) 2 quinone and succinate originally present. 3 malonate and succinate originally present. 4 quinone originally present, succinate added 35 minutes later (rate during next 10 minutes not plotted). 5 malonate originally present, succinate added 35 minutes later (rate during next 10 minutes not plotted). 6, malonate, quinone, and succinate originally present. 7 malonate and quinone originally present, succinate added 35 minutes later (rate during next 10 minutes not plotted).

for the quinone to attack, that is the malonate shields the enzyme against the quinone. Curves 6 and 7 could also be explained by assuming that malonate reacts with quinone directly and thereby prevents it from exerting its effect. That such is not the case, however, was proved by the following experiment which was suggested by previous experiments with urease (6) showing that this enzyme is very sensitive to quinone: urease was tested with malonate and

quinone since, if the action of malonate in the succinic dehydrogenase system were due to reaction with quinone rather than combination with the dehydrogenase, it should also protect urease against quinone. The results showed that malonate does not inhibit urease and therefore has no affinity for this enzyme, and furthermore, it has no ability to protect urease against the action of quinone.

#### DISCUSSION

The data presented above deal with the inhibition of succinic dehydrogenase by a group of diverse substances which are believed to act by combining with the SH group of the enzyme, and in contrast to these, a compound, malonic acid, which is believed to act by competing for the carboxylic acid affinity points which form the basis of the succinate-enzyme complex. These fundamentally different mechanisms of combination may be shown schematically as in Fig. 1, in which quinone as an example of the SH inhibitors is shown in combination with the enzyme SH group (stage VI), while succinate and malonate combine with the enzyme on the basis of their  $\text{—COOH}$  groups (stages II and V).

Experimentation with inhibitors whose action is mutually exclusive seems to afford one of the most promising approaches now available for the study of the stereochemistry of enzymes. Though other possibilities most certainly may exist, it is difficult to explain the ability of malonate to protect succinic dehydrogenase against quinone in any terms other than those depicted in Fig. 1, in which the SH group is located *between* the carboxyl affinity points in such a way that, when malonate or succinate is combined with the enzyme, the SH group is *shielded* against attack by the SH reagents.

Hopkins *et al.* demonstrated three facts of paramount interest to this problem: (a) malonate will protect active succinic dehydrogenase against inactivation by GSSG, (b) enzyme which has been inactivated by treatment with GSSG may be reactivated by treatment with GSH, and (c) malonate will *not* prevent GSH from reactivating enzyme which has been inactivated by GSSG. These authors were unable to explain these seemingly paradoxical results and stated only, "That malonic acid protects from GSSG so completely and from GSH not at all suggests structural relations which may prove instructive." We believe that their observations constitute a contribution of fundamental importance which our own work confirms<sup>2</sup> and extends. Furthermore, on the basis of the concept illustrated in Fig. 1, it is easy to see why the results of Hopkins were obtained: if the SH group is located between the carboxyl affinity points, it is obvious that neither succinate nor malonate could ap-

<sup>2</sup> We do not present data on reactivation of GSSG-inactivated enzyme by GSH and the effect of malonate upon this reaction because of technical differences between our preparation and that of Hopkins.

proach the carboxyl affinity points (B and B') when the enzyme is combined with an SH reagent while, on the other hand, if the enzyme is combined with succinate or malonate, the SH reagents cannot approach and react with the SH group.

While it is impossible to provide an answer as to the possible nature of the enzyme groupings which constitute the carboxyl affinity points, it is interesting to attempt a reconstruction of a peptide chain containing cysteine as the SH bearer. When this was done with accurate (Hirschfelder) atomic models, it was found that the  $\text{—NH—}$  groups adjacent to cysteine were opposite the carbonyl oxygens of the carboxyl groups while the SH group fell between the  $\text{—CH}_2\text{—}$  groups of succinate. In the case of malonate, the carbonyl oxygens again approximated the  $\text{—NH—}$  groups, but the  $\text{—CH}_2\text{—}$  group was directed away from the SH, and both hydrogen atoms were on the side away from the SH.

The remainder of Fig. 1 is pure speculation but, since the structural relations shown in stages I, II, V, and VI seem to fit so well with the facts, it seems plausible to suggest that the location of the SH of the enzyme is of functional significance, *i.e.*, that it is involved in the actual mechanism of oxidation of succinate as is indicated in stages II, III, and IV. According to this concept, the enzyme would function by oscillating between the  $\text{EnSH}$  and  $\text{EnS}$  forms, rather than between the thiol and disulfide forms. Succinate would be oxidized to the free radical monodehydrosuccinate in the first step of the oxidation, which would proceed in accordance with the principle of compulsory univalent oxidation described by Michaelis and Smythe (21). The electron might pass to the prosthetic group of the dehydrogenase intramolecularly without the intermediate dissociation of the semioxidized succinate. The idea of intramolecular electron transfer through a protein is not new but has been discussed by Muller (22) and by Szent Gyorgyi (23) in general terms. The second electron would be disposed of in the same manner as the first, and the two hydrogen atoms would enter the medium as hydrogen ions. Thus the mediation between the two-electron dehydrogenation of the substrates and the one-electron transfer of the cytochrome system could be accomplished.

Whether the above concept is correct or not will remain for future research to ascertain. At any rate, the fact that the enzyme in the disulfide form (whether this be  $\text{En—S—S—En}$  or  $\text{En—S—S—R}$ ) cannot be reduced by succinate seems, in view of the evidence presented in this paper, no longer to be a sufficient basis for the elimination of the SH groups from the oxidative mechanism of succinic dehydrogenase. The concept that the function of this enzyme involves the alternate oxidation and reduction of  $\text{EnSH}$  and  $\text{En—S}$  may provide the explanation for the rôle of thiol groups in the mechanism of hydrogen transfer which has been the goal of Hopkins since his discovery of glutathione and for which his papers on succinic dehydrogenase appear to have opened the way.

## SUMMARY

1 The mechanism of succinic dehydrogenase action was studied by means of inhibitors

2 The enzyme is inhibited by a large number of diverse compounds whose only common denominator appears to be their ability to react with SH groups. These compounds include quinonoid structures, sulfhydryl reagents, sulfhydryl compounds, copper, zinc, selenite, and arsenite

3 In contrast to the above inhibitors, the action of malonate does not appear to involve sulfhydryl groups and is explained on the basis of its affinity for the enzyme groups which react with the carboxyl groups of succinate

4 The action of malonate and the sulfhydryl reactants is *mutually exclusive*, and this fact suggests the conclusion that the sulfhydryl group of the enzyme is located between the carboxyl affinity points

5 On the basis of the deduced structure of the succinate-activating center of the enzyme, it is suggested that the enzyme may function by oscillating between the EnSH and EnS forms, rather than by a thiol-disulfide equilibrium

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## THE ANTIPROTEOLYTIC ACTIVITY OF SERUM

### I. THE NATURE AND EXPERIMENTAL VARIATION OF THE ANTIPROTEOLYTIC ACTIVITY OF SERUM

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#### I. REVIEW OF THE LITERATURE

##### 1. *Nature and Experimental Variation*

Hildebrandt (1893), Camus and Gley (1897) and Landsteiner (1900) were the first investigators to observe that normal serum markedly inhibits the action of trypsin and of yeast proteases. Opie (1905) and Jochmann (1908) demonstrated its ability to inhibit leucoprotease. Study of numerous species of animals (Launoy, 1919) showed antiproteolytic activity to be a constant property of mammalian and bird serum, but innumerable investigations have as yet led to little success in the identification of the substance or substances responsible for this property.

The earliest, and still prevalent, theory of the origin of serum antiprotease is that it is a protective antibody formed by the body against proteolytic ferments emptied into the blood stream. This idea had its inception in the work of Achalme (1901) and Meyer (1909), who reported an increase in the antiproteolytic<sup>1</sup> activity of serum in response to intraperitoneal injection of trypsin into guinea pigs. This finding was refuted by some investigators (e.g. Bergell and Schutze, 1905, Young, 1918) but confirmed and extended by Jochmann and Kantorowicz (1908), who demonstrated a rise in activity following subcutaneous injection of leucoprotease into rabbits, and who concluded from this that leucoprotease serves as the normal antigen in response to which antiprotease is produced.

Others (e.g., von Bergmann and Gulecke 1910) postulated that the pancreas rather than the leucocytes supplied the hypothetical protease antigen, and offered evidence that the antiproteolytic activity on the serum increases following implantation of the pancreas into the peritoneal cavity.

This seemed to be supported by the work of Coblinier (1910), who showed that serum antiproteolytic activity decreases considerably following extirpation of the pancreas.

Indirect evidence against the antibody theory has come from Landsteiner's re

\* Henry Strong Denison Scholar for 1941-42

<sup>1</sup>The term "antiproteolytic" is here used to represent the ability to inhibit many animal and plant proteases which are active at about neutral pH. Trypsin is the protease used in this and most other experimental work, but inhibition of leucoprotease and of other proteolytic enzymes has repeatedly been demonstrated

peatedly confirmed observation (1900) that antiprotease activity is a property of the albumin fraction of serum, whereas antibody activity is a property of the globulin fraction. Furthermore, there is general agreement that increased enzyme inhibiting action of serum, when it occurs, rarely exceeds three or four times the original degree of inhibition, in contrast to true antibodies, which increase to the extent of thousands of times the original antibody content of the blood in response to injections of antigen.

Impressed by these considerations, and by his discovery that charcoal inhibited trypsin by adsorbing it, Hedin (1906-07) attempted to prove that serum exerted its antiprotease action by adsorption of the enzyme, supposedly by serum albumin. Many of the more recent workers, notably Banting and Garms (1930), Fine (1931), and Chrometzka ("neurohormonal regulation" theory, 1932) have accepted Hedin's idea, largely on theoretical grounds. But Hanson (1918) was unable to find any variation in the concentration of serum protein when the antiproteolytic activity of the serum was doubled, or even tripled.

A third group of workers followed the lead of Schwartz (1909) and Bauer (1910), who observed that after serum antiproteolytic activity had been "extracted" with ether, it could be restored by the addition of lipoids to the serum. This group attempted to prove (Jobling and Petersen, 1914) that the antiproteolytic activity of serum is due to its content of unsaturated fatty acids. They demonstrated that unsaturated fatty acids have an inhibitory action on trypsin or leucoprotease which is proportional to their iodine values and which can be destroyed by saturating their double bonds. But that lipoids are not concerned in the antiproteolytic power of serum was shown by Meyer (1909), Cobliner (1910), and Teale and Bach (1919-20). They demonstrated that lipid solvents destroyed rather than extracted serum antiprotease, that lipid solvents had no such effect on dried serum, and that the kinetics of the action of unsaturated fatty acids on trypsin is very different from that of serum.

A fourth line of investigation was opened by Bayliss' discovery (1904), (confirmed by Abderhalden and Gigon (1907)) that trypsin is inhibited by the products of protein digestion. Bayliss assumed that amino acids were responsible. This led Rosenthal (1910) to suggest that serum owes its antiproteolytic action to its amino acid content ("amino acid theory"). But Walters (1912) and Northrop (1921-22) showed that the amino acids themselves have little, if any, effect on tryptic action. Northrop demonstrated that some other constituent of the digest mixture, (unidentified, but apparently a polypeptide), is responsible, combining reversibly with trypsin in accordance with the law of mass action. Shortly afterwards (1922-23) Hussey and Northrop observed that the antitrypsin of normal serum behaves quantitatively like the trypsin inhibitor produced during the tryptic digestion of proteins. More recently Northrop and Kunitz (1932-33) have isolated a polypeptide trypsin inhibitor from the pancreas, and Schmitz (1938), employing their method, has extracted from beef blood small amounts of a substance resembling pancreatic trypsin inhibitor.

## 2 *Physiological and Pathological Variation*

Widespread attention was first drawn to variations in the antiproteolytic activity of the serum by the observation of Brieger and Trebing (1908) that this activity increases markedly in the presence of malignant tumors. This was repeatedly con-

firmed, and was, for a time unfortunately used as a diagnostic test for malignancy. However it was not long before a similar increase was observed in many cases of acute infection, especially if accompanied by high fever (Hort, 1909) anemias (Brenner 1909) late syphilis (Fuerstenberg and Trebing 1909), tuberculosis and hyperthyroidism (Waelli 1912), organic diseases of the central nervous system (Bolten, 1918), anaphylaxis (Pfenfner and Jansch, 1913) protein shock therapy (Jobling and Petersen, 1914) x ray irradiation (Hertzger 1924) pregnancy (Gräfenberg 1909), especially when complicated by eclampsia, after very severe muscular exercise (Prell, 1912) and just before menstruation (Damel and Florian, 1935-36). An increase after meals and a decrease during starvation have been reported by Glaessner (1903) Rosenthal (1910) and Remedi and Bolognesi (1911). The significance of these variations in the antiproteolytic activity of the blood has been little understood although speculation has been rife. It has been regarded by many as an indicator of cachexia especially in chronic diseases.

## II. DATA ON EXPERIMENTAL VARIATION

### 1 *Determination of Antiproteolytic Activity*

It has been shown (Robertson, 1912) that the refractive index of a solution of sodium caseinate is unaltered by tryptic digestion. It is possible to estimate the extent of hydrolysis in such a solution by precipitation of the undigested casein and determination of the refractive index of the filtrate. The amount of casein digested is proportional to the difference between the index of refraction of this filtrate, and that of a control filtrate, from which the casein is precipitated at once.

The antitryptic action of a given serum may be measured by its suppression of casein digestion as compared with a similar solution in which the serum is replaced by saline (Robertson and Hanson, 1918).

The contribution of the serum protein to the substrate (a factor neglected by Robertson and Hanson) may be corrected for by a third digest solution which contains an equal amount of serum whose antitryptic activity has been entirely destroyed by heat (serum diluted and heated at 80°C for 10 minutes). The contribution of the trypsin to the index of refraction (aside from its enzymatic activity) may be corrected for by adding trypsin to the control whose casein is precipitated at once.

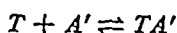
The refractometric measurements indicate the extent of protease but not of peptidase activity and hence will allow investigation of the antiprotease action of serum only. However Jobling, Petersen, and Eggstein (1915) have demonstrated the absence of antipeptidase activity by the serum. In fact the serum contains a small amount of active peptidase (Jobling and Strouse 1912).

The formula derived by Robertson and Hanson (1918) for the determination of the antitryptic activity of serum has been found by Fine (1931) and others to provide only an approximation of the true value, and to be quite unreliable when the concentration of serum is below 10 per cent.



The following derivation of a formula for antiproteolytic activity, based on Hussey and Northrop's convincing demonstration (1922-23) that tryptic protease and its inhibitor in serum combine reversibly in accordance with the law of mass action (the inhibitor serving as a "buffer" of the enzyme), may perhaps more accurately serve the desired purpose

If we assume that one molecule of tryptic protease ( $T$ ) combines reversibly with one molecule of serum antiprotease ( $A'$ ) to form one molecule of protease-antiprotease compound ( $TA'$ )



By the law of mass action

$$\frac{C_T \times C_{A'}}{C_{TA'}} = K$$

If  $E$  = concentration of total trypsin (free and combined)

$T$  = concentration of free (and hence active) trypsin

$A$  = concentration of total antiprotease ( $C_{A'} + C_{TA'}$ )

We may rewrite the equilibrium equation as

$$\frac{(T)(A - (E - T))}{E - T} = K$$

or

$$A = (E - T) \left( 1 + \frac{K}{T} \right)$$

The value of  $E$  is known  $T$  is calculated from the experimental results with the aid of Schutz's "law"

$$X = k\sqrt{T} \text{ (at constant incubation time)}$$

where  $X$  = amount of protein digested

and  $T$  = concentration of free enzyme

Table I illustrates the calculation The total volume of each solution was 3 cc In each serum-added series the serum was at 1.33 per cent concentration All mixtures were at pH 8 (phosphate buffer), and were incubated for 4 hours at 37°C The undigested casein of each was precipitated with acetic acid and filtered off The index of refraction of each filtrate was then determined These readings were corrected as previously described

It is seen that complete digestion of 50 mg/cc of sodium caseinate resulted in an increase in the index of refraction of the solvent of 0.0064 units, so that an increase of 0.0001 unit corresponds to the digestion of 0.78 mg/cc of casein This enables easy calculation of  $X$ , the concentration of digested casein, and

of  $\frac{X}{\sqrt{E}}$ , which is seen to be constant, (until excess enzyme was added) Since  $\frac{X \text{ control}}{\sqrt{E}} = \frac{1 \text{ serum}}{\sqrt{T}}$ ,  $T$  is readily calculated for each concentration of enzyme. This leaves two unknowns in the equilibrium equation,  $A$  and  $K$ .  $A$  is constant for any one serum so that values for  $E$  and  $T$  can be entered for two different enzyme concentrations (calculated for  $T > 0.0002$  mg/cc., since concentrations of active enzyme smaller than this were considered negligible), and  $K$  determined by eliminating  $A$  by subtraction. This was done for several sera, and  $K$  was relatively constant at 0.0018. Using this value for  $K$ ,  $A$  was calculated for these and other sera, and was constant for each serum (within

TABLE 1  
*The Effect of Serum on Varying Concentrations of Trypsin*

(E) Con- centration of crude trypsin	Con- centration of casein	Control (no serum)			Serum 1 (1.13 per cent)				Serum 2 (1.13 per cent)			
		I.R.	(T) Di- gested casein	$\frac{X}{\sqrt{E}}$	I.R.	X	T	A	I.R.	X	T	A
mg./ cc.	mg./ cc.		mg./ cc.									
1.0	50	1.3338	0	—	1.3338	0	—	—	1.3338	0	—	—
2.0	0.17	50	1.3360	17.6	135	1.3339	0.78	0.00036	—	1.3338	0	—
3.0	0.33	50	1.3370	25.0	139	1.3340	1.6	0.00014	—	1.3340	0.00014	—
4.0	0.67	50	1.3383	35.1	135	1.3340	1.6	0.00014	—	1.3340	0.00014	—
5.0	1.33	50	1.3400	48.4	134	1.3350	9.4	0.005	0.179	1.3358	15.6	0.013
6.0	1.67	50	1.3402	50	122	1.3360	14.8	0.014	0.172	1.3368	23.4	0.031
7.0	2.00	50	1.3402	50	113	1.33025	0.034	0.176	1.3380	32.7	0.060	0.144

\* Index of refraction

the error of the index of refraction—about 5 per cent) regardless of the concentration of enzyme.

$A$  represents the concentration of serum antiprotease in the digest mixture. It cannot be expressed in grams or other definable unit. The law of mass action is defined in molarity, but since it is not possible to accurately express concentration of trypsin or casein in moles, milligrams per cubic centimeter were used throughout, resulting in a change in the constants but not in the validity of the equation. The concentration of antiprotease in the serum can be represented as

$$S = \frac{A}{C}$$

where  $S$  = concentration of antiprotease in serum (units/cc.)

$A$  = concentration of antiprotease in digest mixture (units/cc.)

$C$  = cc. of serum in 1 cc. of digest mixture

TABLE II

*The Antitryptic Activity of Varying Concentrations of Serum (Control As in Table I)*

<i>E</i>	Casein	Serum 3	I R	<i>T</i>	<i>A</i>	
<i>mg/cc</i>	<i>mg/cc</i>	<i>per cent</i>				
0.067	50	13.33	1.3340	0.00014	—	
0.067	50	6.67	1.3340	0.00014	—	
0.067	50	3.33	1.3340	0.00014	—	
0.067	50	1.67	1.3340	0.00014	—	
0.067	50	0.83	1.3358	0.014	0.054	0.00014
0.067	50	0.42	1.3371	0.039	0.028	0.00014
0.067	50	0.21	1.3376	0.052	0.015	0.00014
0.133	50	1.33	1.3375	0.049	0.085	0.00014
0.200	50	1.33	1.3394	0.113	0.087	0.00014

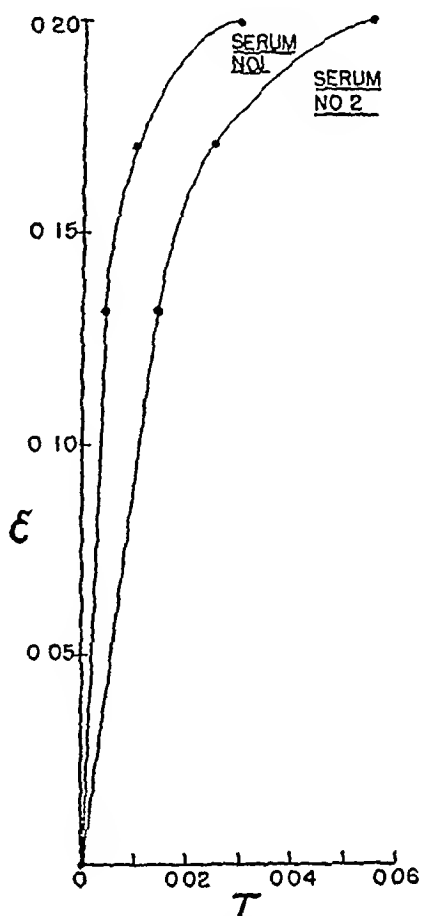
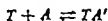


CHART 1 The effect of serum on trypsin

Table II illustrates the approximate constancy of *S*, regardless of the concentration of serum (provided that the change in the index of refraction is g

than the limit of accuracy of the measurement) Measurements over a wider range, however, show relatively more activity at low concentration than at high, in accordance with the law of mass action.

In Chart 1, the values for  $T$  and  $E$  given in Table I are plotted against each other Similar curves, of varying initial slopes, were obtained for the many other sera studied It is evident that the extent to which  $\frac{\Delta E}{\Delta T}$  ( $\pm e$ , the slope of the curve) exceeds 1 is dependent on the concentration of antiprotease present. When  $T$  is small compared to  $E$ ,  $\pm e$  when small amounts of trypsin are acting in the presence of serum,  $(E-T)$  approaches  $E$ , and  $A$  becomes proportional to  $\frac{E}{T}$ , producing a straight line curve whose slope  $\left(\frac{E}{T}\right)$  is proportional to the concentration of antiprotease As  $E$  increases,  $T$  increases, and the reaction



is driven toward the right The concentration of free antiprotease approaches zero, and the curve approaches a straight line with a slope of 1, increments of  $E$  producing equal increments of  $T$  The shape of the curve reflects the fact that the greater the concentration of trypsin, the less is the per cent inhibition, despite the higher total concentration of trypsin inhibited

The calculation of  $S$  with the aid of the above formula is not limited to the refractometric method of following digestion but should be equally applicable to any method that measures protease activity

## 2 Procedure and Results

Reports on the variation of antiproteolytic activity in response to injection of trypsin have been far from unanimous This may in large part be due to failure to appreciate that normal variations in antiproteolytic activity are constantly occurring that different samples of equally active crude trypsin are frequently inhibited to different extents by the same serum (Young, 1918), and that the choice of method has a very important effect on the results obtained (Fine, 1930)

Hanson (1918) reported that the normal variation in antitryptic activity of the serum of several rabbits over a period of weeks was approximately 50 per cent. He also reported that subcutaneous injection of large amounts of crude trypsin resulted in a marked fall in the antitryptic activity, followed, if injection was continued, by a rise within a few days. This rarely exceeded three times the average normal variation during a similar period Then, despite continued injection, a rapid return to the normal range occurred. These results have been confirmed by some workers, and refuted by others

The effect on the antitryptic activity of the following procedures was studied, and it was hoped that this might not only indicate the existence and magnitude

of a response to trypsin, but might also furnish a clue to the mechanism of the action of trypsin and to the nature of the antitryptic substance (1) Intramuscular injection of trypsin (2) Intravenous injection of trypsin (3)

TABLE III

*Daily Normal Variation in Antiproteolytic Activity*

Day	1	2	3	4	5	6	7	Average daily variation	
								Units	Per cent
Rabbit 1	6 0	7 1	7 6	8 1	6 3	5 7	5 2	0 8	12
" 2	3 5	4 8	4 5	3 8	2 8	3 0	4 2	0 8	21
" 3	5 3	5 1	4 5	4 9	3 5	4 7	4 8	0 7	15
" 4	6 8	6 6	8 2	6 8	5 6	5 0	5 7	0 95	15

TABLE IV

*Response to Intramuscular Injection of Trypsin (25 Mg /Day, 2.5 Cc of 1 Per Cent Solution in 0.85 Per Cent NaCl, Seitz Filtered, Sterile)*

Rabbits 1-6 Daily injection of active trypsin for 52 days

7 and 8 Daily injection of active trypsin for 30 days

9 and 10 Daily injection of denatured trypsin for 52 days

Before injection			Daily injection								After injection					
Day	7	4	1	1	6	11	16	21	26	31	6/36	9/39	16/46	22/52	26/4	29/7
Rabbit 1	6 0	7 6	5 2	5 8	6 7	6 5	7 1	8 6	12 2	13 0	10 2	8 8	8 6	9 0	7 1	5 2
" 2	3 5	3 8	4 2	4 0	3 5	5 2	7 3	9 0	13 1	13 5	9 8	8 5	8 2	8 8	6 4	5 1
" 3	5 3	4 9	4 8	5 5	6 2	6 0	6 9	9 3	11 6	13 3	10 8	9 0	9 1	9 5	7 2	4 7
" 4	6 8	6 8	5 7	5 0	5 3	6 3	6 1	9 8	13 6	13 3	12 1	10 0	11 0	10 8	8 2	7 0
" 5	5 2	—	6 2	6 6	—	6 8	—	7 6	—	11 0	9 1	8 0	Died		—	—
" 6	5 8	—	6 0	6 7	—	7 0	—	7 3	—	10 8	8 8	7 9	8 4	8 5	5 6	7 0
" 7	7 2	—	8 1	9 1	—	9 6	—	10 3	—	14 0	8 1	7 0	7 4	—	—	—
" 8	6 5	—	5 7	6 0	—	6 4	—	8 2	—	12 9	6 6	6 2	5 8	—	—	—
" 9	5 7	—	5 3	5 0	—	4 2	—	6 0	—	4 9	—	6 7	—	5 8	—	—
" 10	6 0	—	6 6	6 2	—	7 5	—	7 2	Died	—	—	—	—	—	—	—

Oral administration of trypsin (4) Denatured trypsin, (a) administered intramuscularly, (b) administered intravenously, (c) administered orally

In addition, the local reaction to trypsin injection was studied at different blood levels of antiproteolytic activity

The results obtained are recorded in Tables III-VII Antiproteolytic activity is expressed in S units/cc of serum Crude trypsin (Fairchild) was used throughout

TABLE V

*Response to Oral Administration of Trypsin (200 Mg / Day via Stomach Tube)*

Rabbit 1-4 Daily administration of active trypsin for 4 days.

5 and 6 Daily administration of active trypsin for 8 days.

7 and 8 Daily administration of denatured trypsin for 4 days.

Before		Daily administration						After		
Day	1	1	2	3	4	2/6	4/8	6/2	9/3	12/8
Rabbit 1	8 7	8 3	10 3	10 5	12 0	12 5	10 9	10 3	7 9	—
2	6 0	6 5	6 8	10 3	11 6	12 6	10 6	7 5	7 2	—
3	7 5	8 3	9 8	10 8	14 8	10 2	9 3	8 0	6 4	—
" 4	7 0	6 8	10 3	11 5	10 6	8 1	7 0	7 2	6 1	—
5	7 7	7 9	10 4	10 1	14 8	14 6	14 4	14 0	8 3	8 5
6	5 0	5 8	6 3	8 9	12 7	13 1	12 5	12 0	8 7	4 2
7	6 6	7 5	7 3	8 5	6 8	7 6	—	—	—	—
" 8	6 2	5 5	6 3	4 9	5 3	7 1	—	—	—	—

TABLE VI

*Response to Intravenous Injection of Trypsin (50 Mg / Day)*

Rabbits 1-3 Daily injection of active trypsin for 15 days.

4 and 5 Daily injection of denatured trypsin for 15 days.

Before		Daily administration					After
Day	1	1	3	7	10	13	2
Rabbit 1	7 6	8 0	8 9	8 6	8 0	9 1	8 2
" 2	7 0	6 1	5 5	Died	—	—	—
3	5 8	6 4	4 7	7 3	8 1	8 3	7 7
4	4 5	5 1	5 8	4 9	5 6	4 0	3 4
" 5	4 3	6 2	Died	—	—	—	—

TABLE VII

*Local Reaction 24 Hours after the Subcutaneous Injection of Trypsin (25 Mg) (2.5 Cc of 1 Per Cent Solution in 0.85 Per Cent NaCl Salts Filtered) at Different Blood Levels of Antiproteolytic Activity*

Rabbit 1			Rabbit 2		
Day	S	Size of resulting ulcer (mm. <sup>2</sup> )	Day	S	Size of resulting ulcer (mm. <sup>2</sup> )
1	4 3	250	1	9 6	70
4	7 0	30	3	6 9	320
5	9 3	18	13	11 2	6
Rabbit 3			Rabbit 4		
Day	S	Size of resulting ulcer (mm. <sup>2</sup> )	Day	S	Size of resulting ulcer (mm. <sup>2</sup> )
1	3 8	1000	1	8 8	240
13	9 3	250	11	10 8	130
19	8 0	320	17	12 0	90

## III DISCUSSION

The results recorded in the tables are reproduced in part in Charts 2 to 4, and they show the following

1 Daily variations in antiproteolytic activity of approximately 20 per cent may be considered to be normal, so that experimental variations must exceed this in order to be significant There is not sufficient data to evaluate the

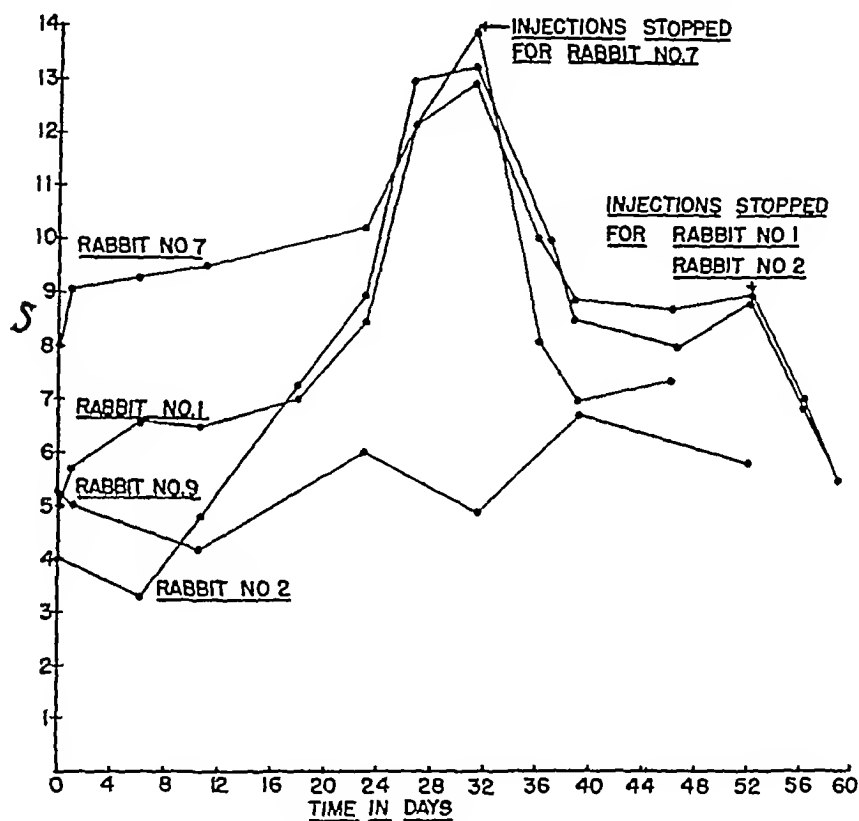


CHART 2 Response to intramuscular injection of trypsin

average variation over days and weeks, but there is strong indication that this rarely exceeded 50 per cent of the average value

2 Daily intramuscular injection of a solution of 25 mg of crude trypsin produced little significant change in the antiproteolytic activity for about the first 11 days Following this period the activity gradually increased, reached a maximum (representing an increase of from 70 per cent to 250 per cent over the average normal value) at about the 4th week, and then declined during the next 10 days to a value which was only about 40 per cent to 120 per cent above the average normal, and remained relatively fixed at this level during the next 2 weeks If the injections were stopped when the antiproteolytic

activity was at its height, the activity fell off much more sharply than if injection were maintained, and returned to the normal range within a week. Cessation of injections at the 52nd day also resulted in return to the norm in less than a week. The intramuscular injection of denatured trypsin did not have any significant effect on the antiproteolytic activity of the serum.

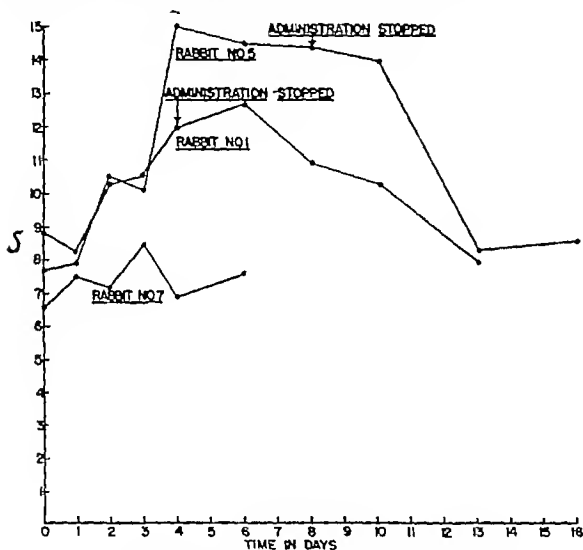


CHART 3 Response to oral administration of trypsin

3 Daily oral administration of 200 mg of trypsin resulted in a rapid rise in antiproteolytic activity within the first day or two, which was maintained during the 4 or 8 days of administration and which returned to the normal range within a week after this was stopped. Denatured trypsin had no such effect.

4 Daily intravenous injection of 50 mg of trypsin produced no variation in activity comparable to intramuscular or oral administration. Denatured trypsin was likewise without effect.

5 The subcutaneous injection of 25 mg of crude trypsin into animals at different blood levels of antiproteolytic activity resulted in the production of



ulcers varying from  $6 \text{ mm}^2$  in area to large sloughs up to  $1000 \text{ mm}^2$  in area. Microscopic examination of the underlying subcutaneous tissues showed in-

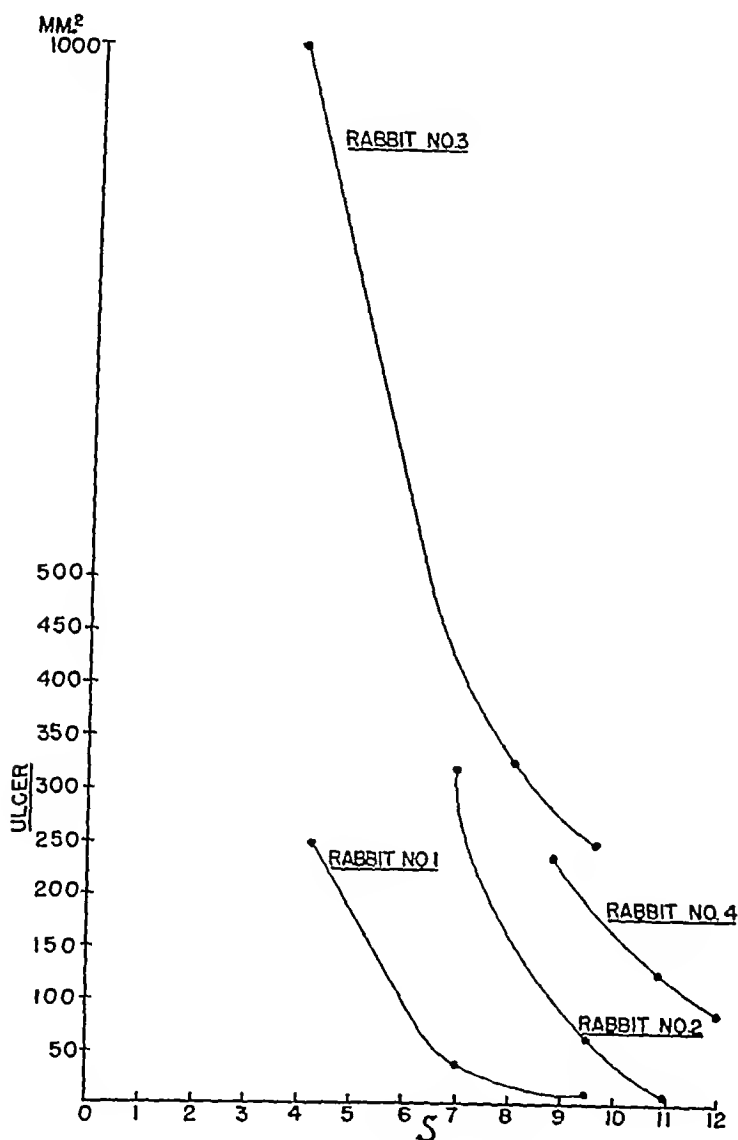


CHART 4 Local reaction to trypsin

flammation and necrosis, corresponding in severity to the size of the ulcer. Comparison between the size of the ulcer and the antiproteolytic activity of the serum shows a rough inverse correlation (in the four animals studied) between the activity of the serum of any one rabbit and the size and severity of the ulcer in that animal, low antiproteolytic activity being associated with a severe local reaction, and *vice versa*.

The experimental results outlined above strongly indicate that an antiproteolytic response to trypsin occurs only when the enzyme is active, and when it is administered to the animal in such a way as to allow enzymatic action to occur. The ineffectiveness of intravenous injection of trypsin could well be explained by the prompt neutralization of the enzyme by the antiproteolytic action of the circulating blood and its maintenance in inactive combination due to the excess of antiprotease. The decline in antiproteolytic activity after a maximum has been reached as a result of daily intramuscular injection could also be explained by decreased proteolysis by the enzyme, here resulting from the increased neutralizing action of the blood, and hence of the exudate that responds to the local necrotizing action of trypsin.

The work of Bayliss (1904) demonstrating that trypsin is inhibited by the products of protein digestion, and of Northrop (1921-22) identifying the trypsin inhibitor as a polypeptide, has already been referred to. The results of experimental variation of antiproteolytic activity that have been described are compatible with this mechanism of antiprotease production. They suggest that the products of protein hydrolysis in the intestine and parenterally are an important factor in the antiproteolytic activity of the serum. These products are most likely polypeptides similar to the trypsin inhibitor produced *in vitro* by Northrop. The rapid increase in serum antiprotease following oral administration of trypsin does not contraindicate a polypeptide inhibitor since the intestinal absorption of peptides, and even of small amounts of larger molecules, is now recognized (Sussman *et al.*, 1928).

If the above mechanism of antiprotease production is true, it would be expected that oral administration of trypsin to animals deprived of food would produce no rise in antiproteolytic activity. Two rabbits that were so studied after 4 days of starvation showed no increase in serum activity after 3 days of trypsin *per os* (200 mg./day). Unfortunately, they died on the 8th day of starvation, so that the experimental results are only suggestive.

The physiological and pathological variations in the antiproteolytic activity of the serum that have been reported are not at variance with a 'proteolytic' theory of the origin of this activity. The postprandial increase in activity and the decrease that occurs during starvation (Glaessner, 1903, Rosenthal, 1910, and Remedi and Bolognesi, 1911) would be attributed to the rôle that intestinal digestion of proteins plays in the production of the antiprotease. The proteolytic enzymes that are abundant in placental tissue (Maeda, 1923) would be responsible for the increase during pregnancy. The increase in hyperthyroidism (Waeli, 1912), in many cases of acute infection with high fever (Hort, 1909), and after very severe muscular exercise (Preti, 1912) would be due to increased protein catabolism which might also result in the formation of antiprotease. That the marked rise accompanying malignancy may well be due to resultant tissue necrosis and autolysis is supported by the observation

of Raab (1935) that the increase of serum antiproteolytic activity in malignant disease is roughly proportional to the degree of disintegration and proteolysis in the tumor. The importance of tissue autolysis in pathological increases of antiproteolytic activity has been emphasized by Meyer (1909), by Braunstein and Kepinow (1910), who increased serum activity by injecting intravenously the products of autolysis of tumors, liver tissue, and other protein-rich substrates, and by Opie, Barker, and Dochez (1911), who showed that substances such as chloroform, which cause necrosis of liver cells, cause a parallel increase in serum antiproteolytic activity. Finally the increased serum antiprotease observed in advanced tuberculosis (Waell, 1912), and in other conditions associated with "cachexia," may perhaps be due to the increased protein catabolism associated with the wasting that is a prominent feature of these conditions.

That the increase in antiproteolytic activity in many of the above conditions might, at least in part, be due to decreased serum protease, rather than increased antiprotease, is contraindicated by the work of Falls (1915) and of Jobling, Petersen, and Eggstein (1915), who have reported that serum protease increases in many of these conditions.

The possibility that antiprotease is an antibody produced in response to trypsin is rendered highly unlikely by the nature, magnitude, and variation of the antiprotease response. Nevertheless, precipitin tests between the serum of rabbits, before and after trypsin injections, and various dilutions of trypsin were performed. Outside of a weak precipitin reaction with serum of animals that had received trypsin intravenously they were all negative, and the positive precipitin reactions showed no parallelism to antiprotease activity. It is furthermore probable that the antibodies that were observed were produced not in response to trypsin, but to other proteins in the crude trypsin, particularly since TenBroeck (1934) was unable to obtain positive precipitin reactions following injection of crystalline trypsin, although he obtained positive Dale anaphylactic tests. And that such antibodies are responsible for the antitryptic activity of the serum and for the ability of injected rabbits to neutralize the local action of trypsin, as was believed by Achalmé (1901) and by Young (1918), seems very unlikely in view of the great dissimilarity between the antiprotease response to trypsin, described above, and the antibody response to the enzyme, described by TenBroeck.

Northrop (1929-30) has discovered that when pepsin is injected into an animal it is almost immediately denatured, so that the antibodies which result are largely antibodies to the denatured pepsin. That this is probably not true in the case of trypsin is indicated by the failure of the precipitin reaction to become any more positive when denatured instead of active trypsin is employed.

Animals to whom trypsin had been previously administered did not show

increased response when they received the enzyme again at a later date (by any route)

A few findings present objections to the "polypeptide" theory of serum antiprotease. Benetato, *et al* (1937) have reported an inverse relationship between serum antiproteolytic activity and serum non protein nitrogen. However, this work is at least partly refuted by the observations of Shono (1933), and, in any event, awaits confirmation. More serious objection is raised by the fact that serum antiprotease is not dialyzable, and is destroyed by heating serum (after dilution to prevent coagulation) at 80°C for 10 minutes (Fujimoto, 1918), neither of which would be expected if it were a simple polypeptide. This would be explained, however if the evidence advanced by Louseleur and Colliard (1937) that polypeptides in the blood are normally adsorbed to the plasma proteins is confirmed, and is shown to apply to serum antiprotease. Attempts which were made to dissociate such a combination by lowering the pH of the serum (or of the plasma, which has the same antiproteolytic activity) below 3, at which pH the compound of trypsin and pancreatic trypsin inhibitor dissociates, resulted in an irreversible loss of antiproteolytic activity. (This may perhaps explain the small yield of crystalline antitrypsin obtained by Schmitz's method of extraction.) Some clue to the situation may be offered by the finding that when crystalline pancreatic trypsin-inhibitor (prepared after the method of Northrop and Kunitz, 1932-33) or crystalline serum antitrypsin (prepared after the method of Schmitz 1938) was heated at 80°C for 10 minutes, it retained its activity, but the addition of a small amount of serum (and to a lesser extent, of serum albumin) resulted in a marked loss of activity on heating. Experiments investigating the effect of serum and of serum albumin on the dialyzability of the above crystalline compounds were unfortunately inconclusive. However, Melln (1932) has reported that he was able to free trypsin from the trypsin-serum antiprotease combination by dialysis of the antiprotease.

Since serum antiprotease is not dialyzable, it should not be freely diffusible in the body except when inflammation, or other pathological process, increases capillary permeability. Such has been found to be the case. Thus Fazio and Chiarolaza (1910) have reported the absence of antiprotease from normal serous fluids and from most transudates, while Weinberg and Laroche (1909) have demonstrated its presence in exudates. Muller (1907) was the first to show the absence of antiprotease from normal cerebrospinal fluid, and Dochez (1909) the first to show its presence during meningitis. The latter's findings have been more recently confirmed by Kaplan *et al* (1939), who also found antiprotease in the cerebrospinal fluid of patients with brain tumors invading the meninges or ventricles. (According to Walter (1929) only such tumors as these increase the permeability of meningeal capillaries.) The presence of a small amount of diffusible antiproteolytic substance in normal urine has been

reported by Fujimoto (1918), while numerous workers (e g , Schippers, 1911) have reported the appearance of large amounts of antiprotease in pathological urine, especially in nephritis

#### IV SUMMARY

1 An equation is derived for the calculation of a constant which, experimental results indicate, may be a more reliable index of the antiproteolytic activity of serum than those equations hitherto used

2 (a) Intramuscular administration of trypsin resulted in a slow rise in the antiproteolytic activity of the serum, followed by a lesser decline (b) Intravenous administration resulted in no appreciable variation (c) Oral administration resulted in a rapid rise, which was sustained during the period of administration (d) Intramuscular, intravenous, or oral administration of denatured trypsin resulted in no appreciable variation (e) The extent of the local necrosis following subcutaneous injection of trypsin varied inversely with the antiproteolytic activity of the serum

3 The experimental results indicate that the products of protein hydrolysis in the intestine and parenterally are an important factor in the antiproteolytic activity of the serum They also indicate that antibodies to trypsin are not an important factor in the antiproteolytic activity of the serum

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## THE ANTIPROTEOLYTIC ACTIVITY OF SERUM

### II. PHYSIOLOGICAL SIGNIFICANCE. THE INFLUENCE OF PURIFIED TRYPSIN INHIBITOR ON THE COAGULATION OF THE BLOOD

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Morawitz (1904) originally suggested, and Eagle and Harris (1937) have presented considerable evidence that, in the course of physiological coagulation, calcium plus thromboplastic substance constitute an enzyme system, analogous to trypsin and to several other proteolytic enzymes. This enzyme system is believed to be responsible for the transformation of prothrombin to thrombin, which itself is thought to be a proteolytic enzyme that accelerates the transformation of fibrinogen to fibrin (as first suggested by Schmidt, 1892).

In view of the evidence for this mechanism, it was suspected that serum antiprotease might normally play some rôle in the process of coagulation. This was investigated by studying the effect on coagulation not only of purified serum antiprotease (prepared after the method of Schmitz (1938), which provided a yield of 40 mg. from 5 liters of pig's blood), but also of crystalline pancreatic trypsin inhibitor (prepared from beef pancreas after Northrop and Kunitz (1932-33)) which is believed by Schmitz to be almost identical with the serum compound, and which can be obtained in far greater amounts.

The effect of varying amounts of 0.5 per cent solutions of these preparations (at neutral pH) on the clotting time of oxalated rabbit's plasma, recalcified with an optimum amount of  $\text{Ca}^{++}$  (as determined in Table I), is recorded in Table II. The clotting time was taken at 37°C. as the time between the addition of the  $\text{Ca}^{++}$  and the formation of a clot firm enough not to move down standard Wassermann tubes (run in duplicate) when these were inverted. It is seen that some substance in these preparations is capable of markedly increasing the coagulation time. Control tubes showed that ammonium sulfate, from which the preparations could not be entirely freed, was not important in this respect.

Table III describes the effect of adding increasing amounts of crude trypsin to plasma to which serum antitrypsin or pancreatic trypsin inhibitor had been added and the resulting solution allowed to stand for 15 minutes before the addition of  $\text{Ca}^{++}$ . (It was found that a time interval elapsed before the anti-

\* Henry Strong Denison Scholar for 1941-42



coagulant action was complete) It is observed that the effect of the trypsin was to progressively neutralize the effect of the trypsin inhibitor preparation

TABLE I  
*Optimum Concentration of  $Ca^{++}$*

Oxalated plasma	1 per cent $CaCl_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent NaCl	Coagulation time
	cc	cc	cc	cc	cc	min
0.5	0.2	—	—	—	0.8	$\infty$
0.5	0.3	—	—	—	0.7	$\infty$
0.5	0.4	—	—	—	0.6	5.2
0.5	0.5	—	—	—	0.5	4.0
0.5	0.6	—	—	—	0.4	6.8
0.5	0.7	—	—	—	0.3	9.1

TABLE II  
*Anticoagulant Action of Purified Antiprotease*

Oxalated plasma	1 per cent $CaCl_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent NaCl	Coagulation time
	cc	cc	cc	cc	cc	min
0.5	0.5	0.0	—	—	0.5	4.0
0.5	0.5	0.05	—	—	0.45	5.1
0.5	0.5	0.1	—	—	0.4	6.3
0.5	0.5	0.2	—	—	0.3	9.0
0.5	0.5	0.3	—	—	0.2	22
0.5	0.5	0.4	—	—	0.1	57
0.5	0.5	0.5	—	—	0.0	20 hrs
0.3	0.3	—	0.0	—	0.4	4.5
0.3	0.3	—	0.1	—	0.3	6.0
0.3	0.3	—	0.2	—	0.2	8
0.3	0.3	—	0.3	—	0.1	11
0.3	0.3	—	0.35	—	0.05	20
0.3	0.3	—	0.4	—	0.0	120

Table IV serves as a control to show that the addition of trypsin (in the amounts employed) to oxalated plasma plus  $Ca^{++}$  of itself only slightly accelerates coagulation. Hence the effect of trypsin described above must be chiefly a specific neutralizing (or destructive?) effect on the anticoagulant.

The addition of trypsin to oxalated or citrated plasma is known to cause coagulation. In Table V is described the effect of adding increasing amounts of trypsin inhibitor to the oxalated plasma plus trypsin. This is seen to have

resulted in a progressive increase in the time required for the plasma to coagulate.

These experimental results strongly indicate that a sufficient concentration of serum antitrypsin or pancreatic trypsin inhibitor, prepared as indicated above, strongly inhibits the coagulation of plasma *in vitro*. This effect is largely prevented by the addition of trypsin. That an anticoagulant other

TABLE III  
*Neutralizing Action of Trypsin on the Anticoagulant*

Oxalated plasma	1 per cent $\text{CaCl}_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent $\text{NaCl}$	Coagulation time
	cc.	cc.	cc.	cc.	cc.	min.
0.5	0.5	0.3	—	0.0	0.3	24
0.5	0.5	0.3	—	0.05	0.25	18
0.5	0.5	0.3	—	0.1	0.2	14
0.5	0.5	0.3	—	0.2	0.1	12
0.5	0.5	0.3	—	0.3	0.0	10
0.3	0.3	—	0.3	0	0.3	11
0.3	0.3	—	0.3	0.1	0.2	11
0.3	0.3	—	0.3	0.2	0.1	9
0.3	0.3	—	0.3	0.3	0.0	5.5

TABLE IV  
*Non Influence of 0.1 Per Cent Trypsin on Coagulation by  $\text{Ca}^{++}$*

Oxalated plasma	1 per cent $\text{CaCl}_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent $\text{NaCl}$	Coagulation time
	cc.	cc.	cc.	cc.	cc.	min.
0.5	0.5	—	—	0.0	0.3	4
0.5	0.5	—	—	0.1	0.2	4
0.5	0.5	—	—	0.2	0.1	3.8
0.5	0.5	—	—	0.3	0.0	3.6

than a trypsin inhibitor was present in one or both of the preparations is of course possible, but is contradicted by the neutralizing effect of trypsin.

In an attempt to determine the mode of action of the anticoagulant varying amounts of pancreatic trypsin inhibitor were added to thrombin (prepared by adding thromboplastin (after Mills and Guest 1921) and  $\text{Ca}^{++}$  to prothrombin (after Mellanby 1931)), a time interval of 15 minutes allowed to elapse and fibrinogen (salted from plasma with sodium chloride) added. Table VI indicates the absence of any appreciable antithrombic effect by the trypsin inhibitor.

The "antiprothrombic" effect of pancreatic trypsin inhibitor was investigated by adding varying amounts to prothrombin plus thromboplastin, and adding calcium after 15 minutes. Then, after 2, 5, and 10 minutes a given

TABLE V  
*Neutralization of Tryptic Action by Purified Antiprotease*

Oxalated plasma	1 per cent CaCl <sub>2</sub>	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent NaCl	Coagulation time
	cc	cc	cc	cc	cc	min
0.5	—	0	—	0.4	0.4	9
0.5	—	0.1	—	0.4	0.3	10
0.5	—	0.2	—	0.4	0.2	12
0.5	—	0.3	—	0.4	0.1	14
0.5	—	0.4	—	0.4	0	17
0.3	—	—	0	0.4	0.4	8
0.3	—	—	0.1	0.4	0.3	8
0.3	—	—	0.2	0.4	0.2	9
0.3	—	—	0.3	0.4	0.1	10
0.3	—	—	0.4	0.4	0	13

TABLE VI  
*Lack of Antithrombic Activity of Trypsin Inhibitor*

Fibrinogen (in 0.85 per cent NaCl)	Thrombin (in 0.85 per cent NaCl)	0.5 per cent Pancreatic trypsin inhibitor	0.85 per cent NaCl	Coagulation time
cc	cc	cc	cc	min
0.5	0.4	—	0.3	2.6
0.5	0.3	—	0.4	3.1
0.5	0.2	—	0.5	5.0
0.5	0.1	—	0.6	11
0.5	0.05	—	0.65	30
0.5	0.4	0.3	0	3.0
0.5	0.3	0.3	0.1	3.5
0.5	0.2	0.3	0.2	5.4
0.5	0.1	0.3	0.3	10
0.5	0.05	0.3	0.35	32

amount of each mixture was tested for thrombic activity (Mellanby, 1917) by adding it to a solution of fibrinogen and determining the time that elapsed before coagulation took place. Table VII shows that the trypsin inhibitor markedly inhibited the conversion of prothrombin to thrombin by the action of thromboplastin plus Ca<sup>++</sup>. Whether it did so by acting on the prothrombin, or

thromboplastin, or both, was not experimentally determined. But if the mechanism of coagulation described above is even partly correct, action on the thromboplastin is strongly indicated. That the inhibitor does not act by reducing the concentration of ionized calcium was easily proved by demonstrating that the presence of excess  $\text{Ca}^{++}$  would not neutralize the effect of the anti coagulant. In fact, it resulted in a further prolongation of the coagulation time.

Various workers have suggested that in addition to their probable rôle in the coagulation process proteolytic enzymes are responsible for thrombinolysis,

TABLE VII  
*Antithrombotic Activity of Trypsin Inhibitor*

	Prothrombin (in 0.85 per cent NaCl)	1 per cent CaCl <sub>2</sub>	Thrombo- plastin (in 0.85 per cent NaCl)	0.5 per cent Pancreatic trypsin inhibitor	0.85 per cent NaCl	Coagulation time
	cc.	cc.	cc.	cc.	cc.	min.
A	0.5	0.1	0.1	0	0.3	—
B	0.5	0.1	0.1	0.3	0	—
Fibrinogen						
cc.						
0.5 + 0.1 cc. of A after 2 min incubation						50
0.5 + 0.1 cc. of A after 5 min incubation						24
0.5 + 0.1 cc. of A after 10 min. incubation						3
0.5 + 0.1 cc. of B after 2 min. of incubation						>24 hrs.
0.5 + 0.1 cc. of B after 5 min. of incubation						120 min
0.5 + 0.1 cc. of B after 10 min. of incubation						10 min.

fibrinolysis, and syneresis. Inhibition by purified antiprotease would afford evidence that proteases are involved in these processes.

### *Hemophilia*

Almost all the current theories on the etiology of hemophilia agree in attributing the prolonged coagulation time observed in this condition to a delay in the formation of thrombin. That a deficiency of active thromboplastic substance may be the cause of this is indicated by the fact that hemophilic blood can be made to coagulate in normal time by adding additional platelets (normal or hemophilic), or by adding trypsin (Tyson and West, 1937, Ferguson and Erickson, 1939). In view of these findings, and of the evidence of Addis (1911) and of Eagle (1935) that the platelets and prothrombin content of hemophilic blood are normal, the possibility that an inhibitor of thromboplastin is responsible for the delayed activation of prothrombin to thrombin must be considered.

Evidence that, if such a thromboplastin inhibitor is present in hemophilic

blood, it does *not* possess antiproteolytic activity, was supplied by examination of the plasma and serum of two hemophiliacs (one of whom was studied through the courtesy of Dr W H Howell) In both of these the antiproteolytic activity was in the normal range, despite greatly prolonged coagulation time

### *Heparin*

If we assume serum antiprotease to be a circulating anticoagulant the possibility of its identity with one of the known circulating anticoagulants must be considered

It has been found by independent investigators that anaphylactic shock or peptone injection results in an appreciable increase in the antithrombin content of the serum as well as in its antiproteolytic activity (Jobling, Petersen, and Eggstein, 1915) In addition, both antithrombic (Mellanby, 1908) and

TABLE VIII

	Dog 1		Dog 2	
	Coagulation time	Antiproteolytic activity	Coagulation time	Antiproteolytic activity
	<i>min</i>	<i>S units/cc</i>	<i>min</i>	<i>S units/cc</i>
Before heparinization	20	3 6	17	4 5
During heparinization	115	3 8	90	4 1
(Later) during heparinization	45	3 2	53	4 6

antiproteolytic activity (Fujimoto, 1918) are destroyed by heating plasma or serum at 75° for 10 minutes However, that antiprotease and antithrombin are separate entities is indicated by the experiments of Mellanby and Pratt (1938), and by the evidence presented above that pancreas trypsin inhibitor has little or no antithrombic activity

Recently Jaques and Waters (1940) have shown that heparin is also increased in peptone shock, while other investigators (Glazko and Ferguson, 1940, and Horwitt, 1940) have found that heparin, in sufficient concentration, inhibits the action of trypsin However, some evidence against the identity of heparin and serum antiprotease is supplied by the finding that heparinization does not increase the antiproteolytic activity of the blood

The blood of two heparinized dogs was followed in the Hunterian laboratory through the courtesy of Dr H B Schumacker Table VIII illustrates the lack of any relation found between the heparin content of the blood (as indicated by the clotting time) and the antiproteolytic activity of the serum (The antiproteolytic activity of plasma and of serum are normally equal) The serum

of the heparinized blood was allowed to remain in contact with the alkaline trypsin (crude) for 30 minutes before the casein was added, as suggested by Horwitt. This is a condition which is *not* ordinarily necessary for the antiproteolytic action of serum. Additional experiments showed that the inhibiting action of purified heparin on crude trypsin is much less than the action described by Horwitt on crystalline trypsin. Serum, on the other hand, is equally active against the crude and the crystalline enzyme. Furthermore, as Tyson and West (1937) have shown, trypsin does not accelerate the coagulation of heparinized blood, while it has been known to hasten the clotting of oxalated or citrated blood.

These considerations indicate that while a relatively high concentration of heparin may, *in vitro*, have some antitryptic activity, it is probably not responsible for an appreciable part of the antitryptic activity of the serum.

Horwitt has made the interesting observation that many basic dyes which are powerful anticoagulants are also antiproteolytic. As in the case of heparin this parallelism deserves investigation.

#### SUMMARY

- 1 Serum antitrypsin and pancreatic trypsin inhibitor inhibited the coagulation of plasma *in vitro*
- 2 This could be largely prevented by trypsin.
- 3 The anticoagulant action of the trypsin inhibitor was apparently due to its antiproteolytic action. It had no appreciable antithrombic action.
- 4 Examination of the blood of two hemophiliacs indicated that the prolonged coagulation time of their blood is not due to an excess of trypsin inhibitor
- 5 Examination of the blood of heparinized dogs indicated that heparin does not appreciably contribute to the antiproteolytic activity of the serum

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## THE ANTIPROTEOLYTIC ACTIVITY OF SERUM

### III. PHYSIOLOGICAL SIGNIFICANCE. THE INFLUENCE OF TRYPSIN AND OF ANTIPROTEASE ON BACTERIAL GROWTH AND SULFONAMIDE ACTION

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Sir A. E. Wright reported in 1916 that the addition of trypsin, leucocytes, or leucoprotease to serum permitted the growth of bacteria which would otherwise be unable to grow in serum. His explanation for this finding was that the trypsin inhibited the antiproteolytic activity of the serum and thereby permitted the accumulation of products of protein digestion, which he believed were necessary for the growth of certain bacteria (called by him "serosaprophytes").

Teale has more recently (1933) disputed this explanation, reporting experiments to show that the presence of antiprotease in the serum does not influence bacterial growth and pathogenicity. He has also offered evidence against the theory of Jobling and Petersen (1914) that the toxins produced during bacterial infection, those responsible for anaphylactic shock, and those present in "anaphylotoxin," are all of the same nature and due to the same cause—namely, to the absorption of antiprotease from the serum, and the consequent degradation of protein substances by the uninhibited action of proteolytic enzymes. However, he reported the interesting observation that although an infected animal may die with the antiproteolytic activity of its serum high, low, or unchanged, animals dying rapidly usually possessed low antiproteolytic activity, while those dying slowly usually showed high activity.

The presence of a powerful antiprotease in the blood and in inflammatory exudates, and the presence of considerable leucoprotease in most foci of infection, render important exact information concerning the effect of protease and of antiprotease on the growth of pathogenic bacteria. For this reason the growth of several organisms was followed in serum with (1) antiprotease intact, (2) additional antiprotease added, (3) antiprotease destroyed, and (4) trypsin added, and in peptone, meat infusion, and albumen solution, with and without added trypsin. And in order to investigate the effect of protease and of antiprotease on bacterial growth when inhibited by a sulfonamide drug, the growth of organisms in the above media was also followed in the presence of sulfathiazole (at 10 mg per cent concentration).

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The growth of *Escherichia coli*, *Eberthella typhi*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Streptococcus pyogenes* was followed in the following media

I Serum with antiprotease intact.

1 12.5 per cent rabbit's serum (heated at 56°C for 30 minutes to destroy complement, so as to eliminate the action of normal bactericidal antibodies)

2 1 + trypsin (0.5 mg /cc sterile crude trypsin)

3 1 + sulfathiazole (0.1 mg /cc.)

4 1 + sulfathiazole (0.1 mg /cc ) + trypsin (0.5 mg /cc )

5 1 + pancreatic trypsin inhibitor (2.5 mg /cc.)

(In the following media concentrations are as in I, except where otherwise indicated )

II Serum with antiprotease destroyed

1 12.5 per cent rabbit's serum (heated at 80°C for 10 minutes This completely destroys antiproteolytic activity (Fujimoto, 1918, and by test))

2 Serum + trypsin

3 Serum + sulfathiazole

4 Serum + sulfathiazole + trypsin

III Peptone

1 1 per cent peptone (nutri-peptone,<sup>1</sup> Wilson) (containing 0.5 per cent NaCl and adjusted to pH 7.4)

2 1 per cent peptone + trypsin

3 1 per cent peptone + sulfathiazole

4 1 per cent peptone + sulfathiazole + trypsin

IV Meat infusion

1 2 per cent beef heart (Difco) (containing 0.2 per cent  $\text{Na}_2\text{HPO}_4$  and 0.36 per cent NaCl and adjusted to pH 7.4)

2 2 per cent beef heart + trypsin

3 2 per cent beef heart + sulfathiazole

4 2 per cent beef heart + sulfathiazole + trypsin

V Albumen

1 1 per cent crystalline egg albumen (containing 0.2 per cent  $\text{Na}_2\text{HPO}_4$  and 0.36 per cent NaCl and adjusted to pH 7.4)

2 1 per cent crystalline egg albumen + trypsin

3 1 per cent crystalline egg albumen + sulfathiazole

4 1 per cent crystalline egg albumen + sulfathiazole + trypsin

The growth of the bacteria in these media was followed by measuring the turbidity of the bacterial suspensions at various intervals by means of a photoelectric cell. The opacity of a bacterial suspension of any age is represented as the number of 24 hour old organisms which, when suspended in the same medium, allow the same amount of light to be transmitted. The number of these organisms was determined by direct count of stained bacteria, and a control curve for each organism and each medium was drawn which allowed translation

<sup>1</sup>A mixture of thio-peptone (tryptic "digest" of lung) and medio peptone (tryptic "digest" of casein)

of turbidity measurements into corresponding numbers of 24 hour old organisms. Obviously this translation is not an exact one, since as growth proceeds the size, refractivity, and reflectivity of the bacteria vary in addition to their number. However, the work of Wilson (1926) and of others indicates that the opacity measurement is probably an accurate measure of the total protoplasmic mass. And this is a truer measure of growth than bacterial number. Such possible sources of error as change in the color or opacity of the medium as a result of bacterial action, or merely as a result of incubation, were proved negligible by filtering off the bacteria from control suspensions after varying incubation periods, and determining the opacity of the medium.

The initial concentration of bacteria in each case was approximately 5 million/cc. The organisms were obtained from 5 day old agar slant cultures (to assure appreciable bacteriostatic activity by the sulfathiazole, which, like the other sulfonamide drugs, acts best on older organisms), and were washed in saline before addition to the media.

Examination of the data in Table I and Charts A to E reveals the following

1 *Growth proceeded more rapidly and further in serum that had been heated at 80°C for 10 minutes than in serum that had merely been heated sufficiently to destroy complement*

It cannot be concluded with certainty that this difference is due even in part to the destruction of serum antiprotease, but it seems very likely. It has been repeatedly demonstrated (e.g., Banting and Garms, 1930) that trypsin acts much more readily on the proteins of serum heated sufficiently to destroy its antiproteolytic activity than on the proteins of serum with intact antiprotease. Since bacterial proteases are also inhibited by serum (von Dungern, 1898, Kämmerer, 1911), they too would be expected to digest serum proteins more readily in the absence of serum antiprotease than in its presence.

2 *Growth proceeded more rapidly and further in all media to which trypsin had been added. The increase was greater in the presence of sulfathiazole than in its absence and was most marked in the case of Proteus*

Bainbridge (1911) has found that bacteria (with a few exceptions) are unable to readily digest proteins and perhaps even albumoses and peptones, except when very abundant growth is occurring. He has suggested that they are capable of absorbing only amino acids and lower peptides, and that they can produce proteases only when these protein breakdown products are readily available. This indicates the importance to bacterial growth of the presence of proteases other than bacterial proteases, especially when the concentration of non protein nitrogen is low compared to that of protein, as in the blood and in exudates.

The data reported above show that the growth promoting action of trypsin is also exerted (although to a lesser extent) in peptone and meat infusion, in which the concentration of protein is very low. Since crude trypsin, which

TABLE I

*The Influence of Trypsin and of Antiprotease on Bacterial Growth and Sulfonamide Action*

	Incubation, hrs	<i>E. coli</i> millions/cc					<i>E. typhi</i> millions/cc					<i>Proteus vulgaris</i> millions/cc				
		0	10	20	42	64	0	10	20	42	64	0	10	20	42	64
	<i>Medium</i>															
I	1 Serum	5	130	175	170	170	5	50	140	120	115	5	115	225	225	205
	2 Serum + trypsin	5	140	220	210	185	5	75	275	230	230	5	255	540	540	495
	3 Serum + sulfathiazole	5	50	70	50	10	5	5	25	5	5	5	50	50	50	5
	4 Serum + sulfathiazole + trypsin	5	50	90	95	5	5	45	150	185	165	5	140	185	120	115
	5 Serum + trypsin inhibitor	5	120	130	125	110	5	25	105	100	90	5	70	110	105	105
II	1 Serum heated at 80°	5	310	395	310	290	5	165	160	140	120	5	255	280	210	190
	2 Serum + trypsin	5	210	415	525	345	5	280	410	420	350	5	520	725	565	500
	3 Serum + sulfathiazole	5	105	125	45	45	5	30	50	30	30	5	115	115	135	160
	4 Serum + sulfathiazole + trypsin	5	155	445	385	265	5	95	110	120	130	5	270	630	540	495
III	1 Peptone	(Omitted)					5	45	245	245	240	5	180	260	255	250
	2 Peptone + trypsin						5	50	340	300	240	5	445	560	560	560
	3 Peptone + sulfathiazole						5	35	95	90	75	5	30	280	280	260
	4 Peptone + sulfathiazole + trypsin						5	75	230	235	205	5	45	345	370	285
IV	1 Meat infusion	(Omitted)					5	70	160	115	90	5	140	210	210	210
	2 Meat infusion + trypsin						5	165	330	330	300	5	335	620	625	650
	3 Meat infusion + sulfathiazole						5	85	165	165	120	5	45	195	130	90
	4 Meat infusion + sulfathiazole + trypsin						5	95	180	180	150	5	45	225	375	330
V	1 Albumen	(Omitted)					5	50	95	75	30	5	95	185	140	120
	2 Albumen + trypsin						5	95	190	205	200	5	205	420	460	440
	3 Albumen + sulfathiazole						5	25	25	<1	<1	5	75	25	<1	<1
	4 Albumen + sulfathiazole + trypsin						5	45	45	45	<1	5	95	145	185	165

	Incubation, hrs	<i>Staphylococcus aureus</i> millions/cc					<i>Streptococcus pyogenes</i> millions/cc				
		0	10	20	42	64	0	10	20	42	64
<i>Medium</i>											
I	1 Serum	5	30	120	140	120	5	50	90	115	105
	2 Serum + trypsin	5	30	190	370	360	5	185	165	165	120
	3 Serum + sulfathiazole	5	70	25	5	<1	5	5	5	5	<1
	4 Serum + sulfathiazole + trypsin	5	75	120	120	50	5	50	165	165	140
	5 Serum + trypsin inhibitor	5	30	110	130	120	5	20	45	55	55
II	1 Serum heated at 80°	5	255	250	235	235	5	140	165	165	160
	2 Serum + trypsin	5	455	435	435	345	5	355	355	400	400
	3 Serum + sulfathiazole	5	95	25	25	5	5	25	25	<1	<1
	4 Serum + sulfathiazole + trypsin	5	450	450	380	335	5	140	165	170	170
III	1 Peptone	5	380	380	420	420	5	90	130	130	110
	2 Peptone + trypsin	5	465	485	555	555	5	145	210	220	200
	3 Peptone + sulfathiazole	5	140	250	250	220	5	45	75	75	25
	4 Peptone + sulfathiazole + trypsin	5	320	390	450	450	5	140	95	75	75
IV	1 Meat infusion	5	275	300	380	340	5	100	110	100	95
	2 Meat infusion + trypsin	5	465	465	520	540	5	250	245	240	220
	3 Meat infusion + sulfathiazole	5	230	230	240	245	5	70	45	45	45
	4 Meat infusion + sulfathiazole + trypsin	5	275	375	400	375	5	110	200	205	235
V	1 Albumen	5	95	25	25	<1	5	70	75	85	80
	2 Albumen + trypsin	5	135	225	250	275	5	100	125	120	120
	3 Albumen + sulfathiazole	5	75	5	5	<1	5	25	5	<1	<1
	4 Albumen + sulfathiazole + trypsin	5	120	140	150	170	5	50	80	100	100

was used, contains both protease and peptidase, growth acceleration as a result of peptidase action is indicated in addition to the protease action

Since leucoprotease has tryptic (protease) action its growth promoting properties (especially when the N P N in the medium is relatively low) must be considered. And since serum antiprotease exerts its buffering action against leucoprotease, just as it does against trypsin (Opie, 1905, repeatedly confirmed) it too is very probably important with respect to bacterial growth, but as a growth inhibitor rather than accelerator

The polymorphonuclear leucocytes have been shown by Opie (1922) to be much richer in tryptic leucoprotease than are the mononuclear leucocytes, and to be the chief source of leucoprotease. During acute inflammation they cause

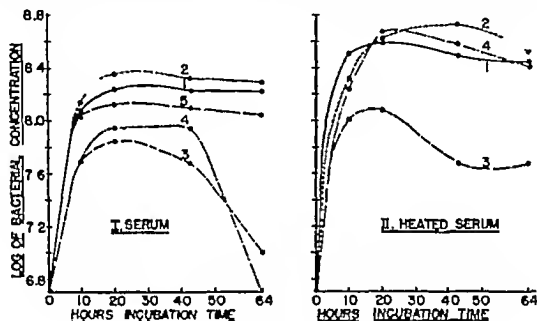


CHART A. Growth of *Escherichia coli*

a considerable accumulation of this enzyme. In diffuse phlegmonous inflammations and in serous cavities the leucoprotease is to a large extent buffered by the simultaneous exudation of fluid rich in serum antiprotease, while in abscess cavities and in cerebrospinal fluid such buffering action is minimal. In acute meningitis the increased meningeal permeability does allow some antiprotease to enter the cerebrospinal fluid, but this does not compare with the far greater amount of leucoprotease that is derived from the polymorphonuclear cells (Kaplan *et al*, 1939). That the uninhibited growth accelerating action of the leucoprotease is at least in part responsible for difficulty of the body, even when aided by chemotherapeutic agents, to control the growth of organisms in acute meningitis, must be considered. Subdural injection of serum unfortunately results in only a temporary increase in the antiproteolytic activity of the spinal fluid lasting not even 24 hours (Dochez, 1909).

In abscess cavities, too, a great preponderance of leucoprotease over anti-

protease occurs, since pressure within the confined space prevents the accumulation of fluid exudate, such as occurs in serous cavities and in phlegmonous inflammations (Opie, 1906) However, here, unlike the cerebrospinal spaces,

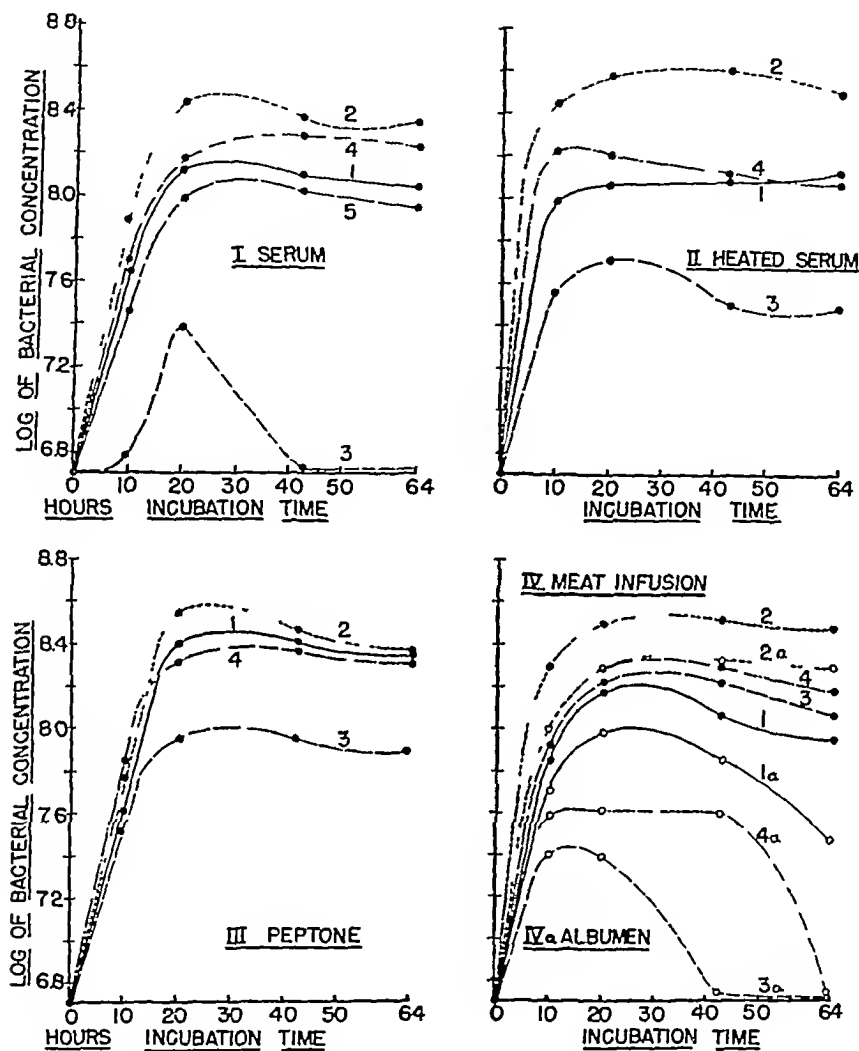


CHART B Growth of *Eberthella typhi*

mechanical factors act to prevent spread of bacteria, and to inhibit their growth by causing a dearth of assimilable food and oxygen, and the accumulation of products of metabolism, including hydrogen ions. The preponderance of protease explains the extensive autolysis that occurs in abscess cavities, but that trypsin will penetrate and digest dead cells only (and probably only dead bacteria, too), and that living cells are not permeable to the enzyme, has been convincingly demonstrated by Northrop (1939). Hence protease would

not be expected to exert any bactericidal action by virtue of its enzymatic activity, although the accumulation of acid products of hydrolysis would, in an unbuffered medium, have both bactericidal and bacteriostatic activity as a

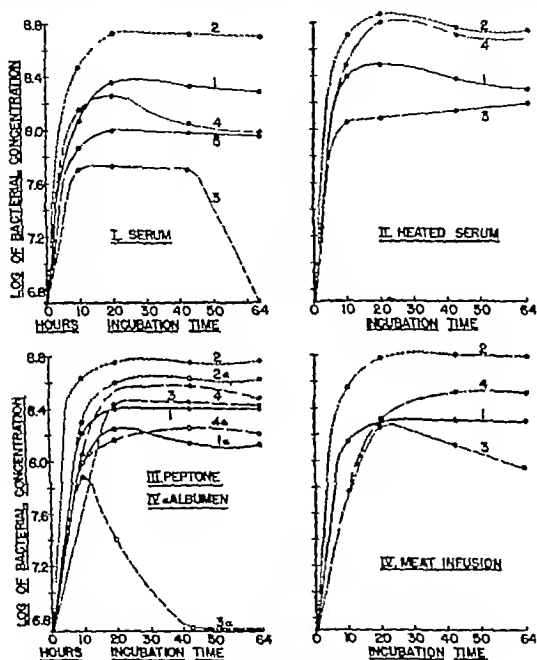


CHART C Growth of *Proteus vulgaris*

result of increase in the concentration of hydrogen ions. The autolytic activity of protease (on dead organisms) is not evident in the experiments described above because the growth promoting influences predominated during the time charted. After longer incubation more rapid decline in turbidity and loss of gram positive staining characteristics occurred in the presence of protease.

3 In a limited number of experiments crystalline pancreatic trypsin inhibitor

somewhat inhibited the growth of all the bacteria studied except *Staphylococcus aureus*. This finding supports the contention that antiprotease can be bacteriostatic, especially in media poor in non-protein nitrogen, and probably as

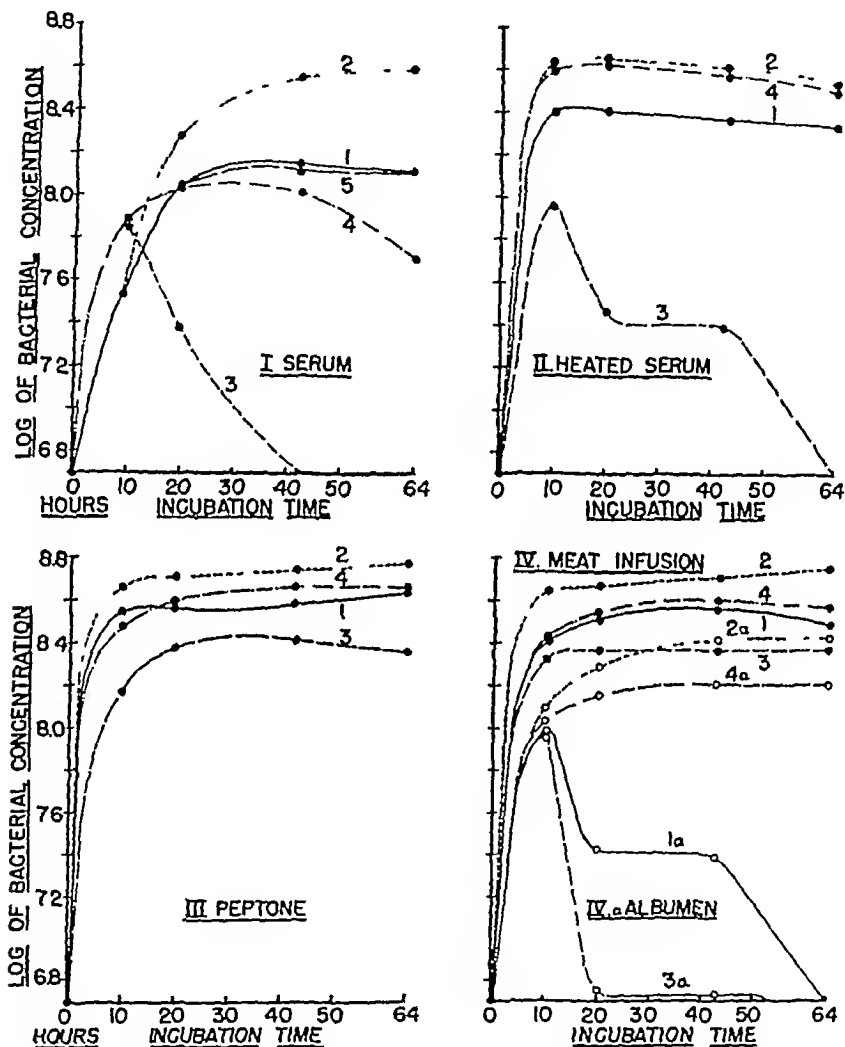


CHART D Growth of *Staphylococcus aureus*

a result of inhibition of bacterial protease. Unfortunately insufficient crystalline inhibitor was prepared to study its action in other media than serum.

That trypsin inhibitor has no bactericidal activity, and trypsin no antibactericidal action, was demonstrated (for *E. typhi*) by incubating organisms (in peptone and in bactericidal serum) with trypsin inhibitor and with trypsin for 2 hours and testing successive dilutions for sterility after the method of Mackie and Finkelstein (1931).

4 The bacteriostatic action of sulfathiazole was

- (a) reduced by destruction of the antiproteolytic activity of the serum (by heat),  
 (b) considerably reduced and sometimes almost obliterated by the presence of trypsin

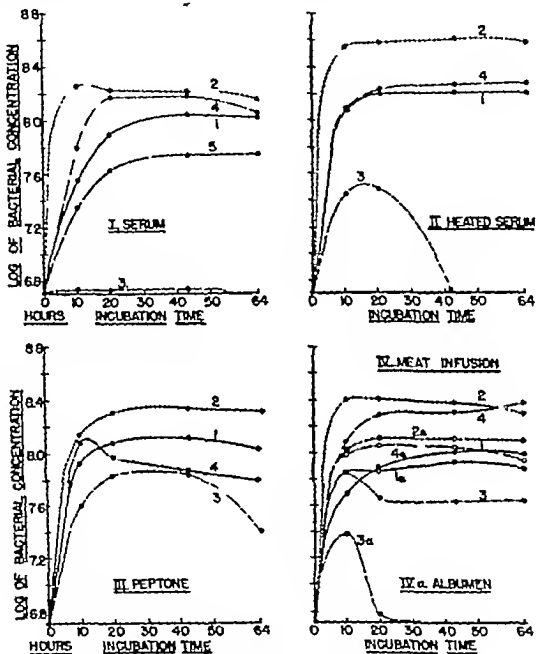


CHART E. Growth of *Streptococcus pyogenes*

in the medium, and (c) greater in serum and albumen than in peptone and meat infusion

Since bacterial growth has been shown to be increased by destruction of serum antiprotease by heat, and by the addition of trypsin, the bacteriostatic action of sulfathiazole in these media would be expected to be correspondingly reduced, as compared to that in unaltered media. The greater bacteriostatic action in serum and albumen than in peptone and meat infusion indicates that



somewhat inhibited the growth of all the bacteria studied except *Staphylococcus aureus*. This finding supports the contention that antiprotease can be bacteriostatic, especially in media poor in non-protein nitrogen, and probably as

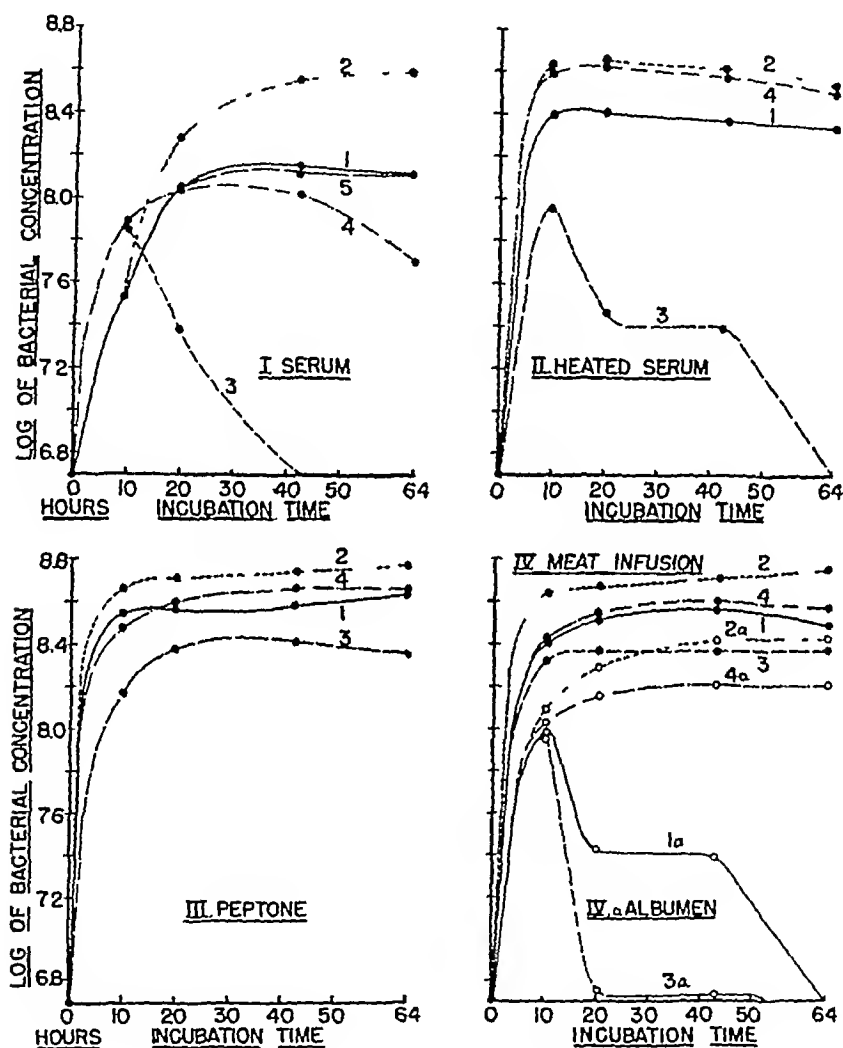


CHART D Growth of *Staphylococcus aureus*

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4 The bacteriostatic action of sulfathiazole in serum was reduced by heating the serum at 80° C, and much more markedly (in any of the media studied) by adding trypsin. It was greater in serum and albumen than in peptone and meat infusion

5 The significance of the experimental results was considered in relation to the possible influence of leucoprotease and of serum antiprotease on bacterial growth and sulfonamide action

#### *Other Physiological Significance of the Antiproteolytic Activity of the Serum*

Holmes, Keefer, and Myers (1935) have presented convincing evidence that the presence of antiproteolytic substances in synovial exudates is of great importance in the prevention of damage to joints by protease liberated from the leucocytes which accumulate in suppurative arthritis

The possible importance of serum antiprotease in the prolongation of insulin action has recently been considered by Horwitz and others. That it may be of significance in this respect is rendered more likely by the demonstration and crystallization from the serum (Schmitz, 1937 and 1938) of small amounts of both protease and antiprotease.

In a series of papers (1935, 1936, 1937) Shute has reported experimental and clinical data designed to show that estrin possesses a special antiproteolytic activity (unlike that of serum antiprotease) which causes, when estrin is present in the blood in excess, abortion, premature labor and abruptio placentae. He has also attempted to prove that the blood concentrations of estrin and of vitamin E are inversely proportional to each other, and that excess of estrin is usually due to vitamin E deficiency. His work has not yet been confirmed.

Rich and Duff have reported (1937) the experimental production of hyaline arteriosclerosis and arteriolonecrosis within 24 hours after the injection of various proteolytic enzymes subcutaneously. Data presented under discussion of the nature and experimental variation of serum antiprotease indicate an inverse relation between the antiproteolytic activity of the serum and the local damage produced by trypsin. The relation (if any) of either of these factors to clinical arteriosclerosis or arteriolonecrosis is not yet known.

The relation of serum antiprotease to the resolution and absorption of inflammatory exudates and absorbable foreign bodies (including catgut), and to organizing pneumonia, has been very little considered.

The use of serum (especially of high antiproteolytic activity) to neutralize the destructive protease liberated in acute pancreatitis and from duodenal fistulae has not yet been investigated.

The experiments of Menkin (1938), and of others, indicate that the increased capillary permeability and leucocyte infiltration characteristic of inflammation are due to a polypeptide formed by proteolysis at the site of inflammation.

the products of protein hydrolysis not only promote bacterial growth, but also inhibit the bacteriostatic action of sulfathiazole

The inhibitory effect of peptone and of various bacterial extracts on sulfanilamide has been known, and has been the subject of intensive study, for the past 4 years (Lockwood, 1938, McIntosh and Whitby, 1939, Stamp, 1939, etc.) Woods (1940) has offered evidence that *p*-aminobenzoic acid is essential for the growth of bacteria, that the enzyme reaction involved in the utilization of this substance is subject to competitive inhibition by sulfanilamide (due to structural relationship between the drug and the "essential metabolite"), and that the inhibitory effect of peptone and of various bacterial extracts is due to their content of *p*-aminobenzoic acid. He and his followers believe that the presence of *p*-aminobenzoic acid preformed in the medium is responsible for the difficulty of inhibiting bacteria by the use of sulfanilamide in peptone and in lesions characterized by considerable tissue destruction. They also believe that the sensitivity of organisms to sulfanilamide action depends on their inability to synthesize *p*-aminobenzoic acid. Recently, Bliss and Long (1941) have produced evidence that another protein breakdown product, methionine, possesses similar antisulfonamide action.

The experimental results recorded above indicate that the products of protein digestion, in addition to inhibiting the bacteriostatic activity of sulfathiazole, also directly promote bacterial growth. That the latter occurs in the presence as well as in the absence of sulfathiazole seems likely. The results indicate the importance with respect to bacterial growth and sulfonamide action of proteolytic enzyme (and peptidase?) in the medium, and therefore the importance of the buffering action of serum antiprotease on this enzyme.

If these conclusions are valid it is to be expected that the growth of bacteria in the body will be more rapid, and the inhibition of sulfathiazole will be greater.

(1) when considerable leucoprotease (and peptidase?) is present, (2) when the organism itself produces active protease (and peptidase?), (3) when the inflammatory exudate is small and poor in antiprotease, (4) when the source of antiprotease (the blood) is poor in this constituent, and (5) when the medium is rich in non-protein nitrogen (especially *p*-aminobenzoic acid?)

#### SUMMARY

1 Heating diluted serum at 80° C for 10 minutes made it a better medium for bacterial growth. This is believed to have been at least partly due to destruction of the serum antiprotease.

2 Growth was accelerated, and proceeded further, in the presence of trypsin.

3 Growth was somewhat retarded in the presence of pancreatic trypsin inhibitor.

# STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

## VII INHIBITION BY RIBONUCLEASE\*

By V R POTTER AND H. G. ALBAUM†

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin Madison)

(Received for publication, January 25, 1943)

Earlier in this series of investigations, it was pointed out that succinic dehydrogenase and cytochrome oxidase are associated with the particulate components of cytoplasm (1), and attention was called to the similarity between these particles and the mitochondria which Bensley (2) separated from broken cell preparations by centrifugal means. Szent Györgyi (3) as well as Stern (4) had previously called attention to the fact that these enzymes are apparently attached to some macromolecular entity. The enzyme which we have called "coenzyme I-cytochrome c reductase" (5) is also in this category (5, 6), and Kabat (7) has shown that alkaline phosphatase is associated with the particulate matter.

During this same period, Claude (8) has been perfecting the method of separation of the morphological components of cytoplasm by centrifugal techniques and has obtained discrete granules of material having "the general constitution of a phospholipid ribonucleoprotein complex" with diameters ranging from 50 to 150  $\mu$ . The particles were considered to pre-exist in the original material and were shown to be similar in size and chemical composition to the particles of the Rous tumor virus.

These independent observations provide no proof, however, that the enzyme systems include a ribonucleoprotein, since the particulate components might consist of a mixture of ribonucleoprotein particles and enzyme particles. Furthermore, there is thus far no evidence that any given particle contains more than one enzyme (9). Isolation of the enzymes in crystalline form does not provide an answer to the question since it seems likely that the highly active enzymes which have been isolated as soluble proteins of low molecular weight may not occur as such in the cell, but rather (4, 7) that they are attached to the larger particles which Claude has shown to contain ribonucleic acid and phospholipid.

Kunitz (10) has recently isolated ribonuclease in crystalline form and has demonstrated that it splits ribonucleic acid into smaller acidic groups without

\* This investigation was aided by the Jonathan Bowman Fund for Cancer Research.

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The possibility of an inhibitory effect of serum antiprotease on this important proteolysis would seem to deserve investigation

I am indebted to Dr J Howard Brown for the facilities to conduct the experiments described in this and preceding papers of this series and for his help and advice, also to Dr W H Howell for criticisms and suggestions which were of great value

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Kunitz (10) has recently isolated ribonuclease in crystalline form and has demonstrated that it splits ribonucleic acid into smaller acidic groups without

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liberating free phosphoric acid. It has no effect upon desoxyribonucleic acid. However, it apparently is able to combine with the ribonucleic acid contained in crystalline tobacco mosaic virus to give an inactive complex as Loring has shown (11). Loring's experiments suggested the investigation herein reported in which it is shown that ribonuclease inactivates the succinoxidase system associated with the particulate components of liver homogenates. A number of other enzymes were also tested in an attempt to learn whether the effect is specific for the succinoxidase system.

#### EXPERIMENTAL

The succinoxidase system was set up on the basis of previous work (1, 9), and its over-all activity was studied by measuring the rate of oxygen uptake in a conventional Warburg apparatus. Both rat and mouse livers and kidneys were used to prepare dilute homogenates, and the homogenization was invariably carried out in ice-cold water which had been recently redistilled from glass, in contrast to our previous method of homogenizing in isotonic phosphate at pH 7.4. In most cases, the distilled water contained sufficient NaOH to give pH 7.8–8.0 so that the dilute homogenates had a pH of 7.0–7.2. Metal contamination was carefully avoided. Two components of the succinoxidase system, succinic dehydrogenase and cytochrome oxidase, were studied in a Cenco photoelectric spectrophotometer on the basis of the change in extinction at 550  $m\mu$ . Coenzyme I-cytochrome *c* reductase was likewise studied on the basis of the change in the cytochrome spectrum, using sodium dihydrocoenzyme I (12) as a substrate. The cytochrome oxidase system was also studied manometrically, using a test system worked out in this laboratory by W. C. Schneider. The ascorbic acid used as a substrate in this work was a gift from Merck and Company. The urease was a commercial preparation, sold by the Arlington Chemical Company, and was studied on the basis of the rate of  $\text{CO}_2$  evolution under conditions modified from those previously described (13). Catalase was studied with dilute liver homogenates as the source of the enzyme. The evolution of oxygen was measured in the Warburg apparatus under conditions employed in this institute by B. E. Kline and H. P. Rusch. Alkaline phosphatase was studied essentially according to Kabat (7), using dilute homogenates as the source of enzyme. We are indebted to K. P. DuBois for testing the adenosine triphosphatase, using liver homogenates under conditions which he has worked out. Xanthine oxidase was studied in the Warburg apparatus, using a highly purified preparation which was a gift from S. W. Schwartzman. Specially purified xanthine was a gift from J. A. Bain. Crystalline, salt-free ribonuclease was obtained through the kindness of Dr. M. Kunitz of The Rockefeller Institute.

In general, the ribonuclease was incubated with the various enzyme systems for 1 to 2 hours at 38° in unbuffered solutions. Since the ribonuclease solutions were acidic, they were brought to pH 6.8–7.0 with 0.002 *N* NaOH immediately before use. The stock solution of ribonuclease was kept in the cold. The pH of the various enzymes was measured with and without ribonuclease, no change occurred either on mixing or upon standing. Since incubation in dilute solution tends to emphasize the destructive action of oxidation and metal contaminants especially in the case of urease

and succinic dehydrogenase, we routinely used two types of controls a "fresh control" to give the original activity and an incubated control to show the effect of incubation in high dilution *per se*. The activity of the "fresh control" was measured simultaneously with that of the other mixtures but during the incubation period it was kept at 0° and in more concentrated solution.

The details of the experimental test systems will be reported with the results, which will be considered under separate headings.

### *Cytochrome Systems*

*Succinoxidase System, Manometric Technique*—Ribonuclease completely inactivates the over-all activity of the succinoxidase system when sufficient time is allowed, if the proper amounts of ribonuclease relative to the amount of homogenized tissue are used, and if the two enzymes are allowed to act in unbuffered solution. Fig 1 shows that the inhibition becomes progressively greater over a 2 hour period. This is in contrast to the results of Loring who reported that the loss in virus activity on standing in contact with ribonuclease was not significantly different from that immediately after mixing. However, it is not clear how the time of incubation could be stated in the virus experiments since the virus-ribonuclease mixture must have remained together for a time after the test leaves were inoculated. Fig 1 also shows that the percentage inhibition varies according to the amount of succinoxidase used since the activity of a small amount of liver is completely destroyed much sooner than the activity of a larger amount. The results indicate that the ribonuclease-succinoxidase relationship is that of an enzyme to its substrate. Fig 2 shows that very small amounts of ribonuclease are able to inhibit the succinoxidase system in unbuffered solution, while much larger amounts are required to produce inhibition in the presence of buffer. In both cases, the inhibition appears to be proportional to the log of the ribonuclease concentration. The effect of buffering is in harmony with Loring's results on the precipitation of a crystalline ribonuclease virus complex. He stated that the precipitate was insoluble in distilled water but could be dissolved readily in phosphate buffer at pH 7.0, in which solution, nevertheless, inactivation of the virus occurred.

*Cytochrome Oxidase, Manometric Technique*—Since the results in Figs 1 and 2 clearly demonstrated the fact that ribonuclease inhibits the succinoxidase system, it was of interest to determine whether the results were due to the inhibition of succinic dehydrogenase or cytochrome oxidase or of both. It was found that both components are inhibited. Cytochrome oxidase was tested by both manometric and spectrophotometric techniques. The manometric tests were done with ascorbic acid neutralized to pH 7.0, as the source of electrons for the reduction of cytochrome *c*. Otherwise, the technique was essen-



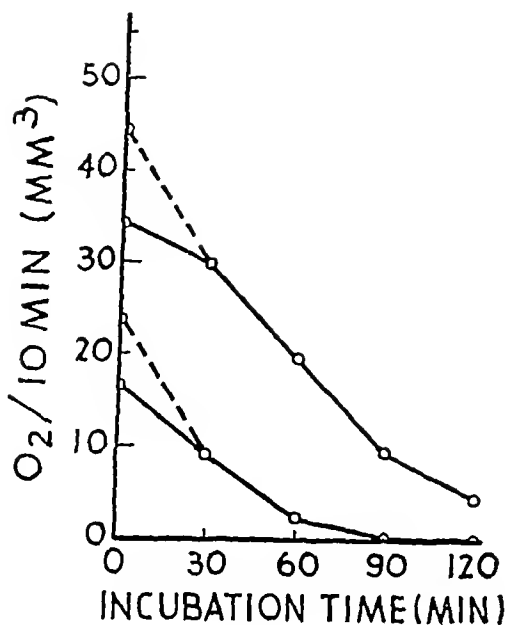


FIG 1

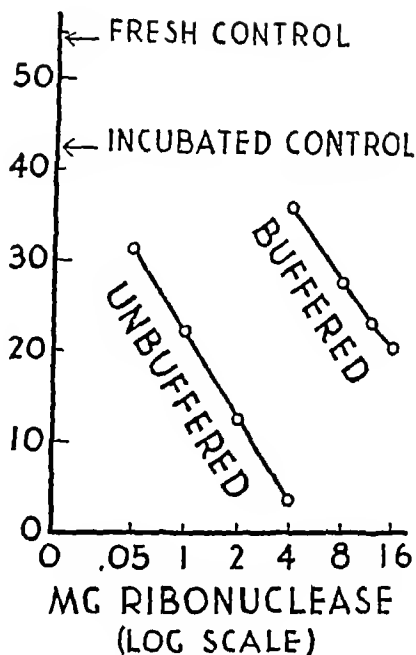


FIG 2

FIG 1 The progressive inactivation of the succinoxidase system by ribonuclease. Each flask contained 1.0 ml of 0.1 M sodium phosphate buffer pH 7.4, 0.4 ml of  $10^{-4}$  M cytochrome *c*, 0.3 ml of  $4 \times 10^{-3}$  M  $\text{CaCl}_2$ , 0.3 ml of  $4 \times 10^{-3}$  M  $\text{AlCl}_3$ , 0.3 ml of 0.5 M sodium succinate, plus water to give a final volume of 3.0 ml after addition of the liver homogenate and ribonuclease. The succinoxidase, in the form of a 5 per cent mouse liver homogenate, was placed in the side arms of the flasks and kept in a warm room for 2 hours prior to the activity measurements. At appropriate times, 0.2 ml of 0.2 per cent ribonuclease was added to the side arms so that the succinoxidase was incubated for 2 hours in all cases but only for the indicated time in the presence of ribonuclease. At the end of the 2 hour period, the flasks were connected to manometers, placed in a thermostat at  $38^\circ$ , the enzyme mixture was tipped into the main flask, and the rate of oxygen uptake was observed for four 10 minute periods. The values at zero time are the controls: the incubated control is on the continuous line, and the fresh control (see text) is on the dotted line. The upper curve is for 10 mg of liver, and the lower curve is for 5 mg of liver.

FIG 2 The effect of various amounts of ribonuclease on the succinoxidase system in presence and absence of buffer. Each flask contained phosphate, cytochrome *c*,  $\text{CaCl}_2$ ,  $\text{AlCl}_3$ , succinate, and water as in Fig 1. All flasks contained 5 per cent mouse liver homogenate equivalent to 10 mg of fresh liver, and 0.2 per cent ribonuclease was added to the flasks in the amounts indicated in the graph. In the "buffered" series, the ribonuclease and succinoxidase were in the main part of the flask with the phosphate, cytochrome,  $\text{CaCl}_2$ , and  $\text{AlCl}_3$ , while the succinate was in the side arms. In the "unbuffered" series, the succinate, etc., were in the main compartment, and the ribonuclease and succinoxidase were in the side arms. The flasks were connected to manometers and shaken in the thermostat for 2 hours. The side arms were then tipped, and the rate of oxygen uptake measured as in Fig 1.

tially similar to that used for the over-all system as in Figs 1 and 2. In a typical experiment, the main flasks contained phosphate,  $\text{AlCl}_3$  and water as in Fig 1, plus 0.5 ml. of 1 per cent ascorbic acid and 0.6 ml. of  $4 \times 10^{-4} \text{ M}$  cytochrome *c*, and the side arms contained 0.3 ml. of 1 per cent mouse liver homogenate plus 0.2 ml. of 0.2 per cent ribonuclease in appropriate flasks. After 90 minutes in the thermostat, during which time the autoxidation rate of the ascorbate was noted, the side arms were tipped into the main compartments, and the rate of oxygen uptake was measured for 10 minute intervals. The results, expressed in  $\mu\text{l. O}_2$  per 10 minutes, were as follows: autoxidation rate, 3.6, fresh control, 58.9, incubated control, 56.8, incubated with ribonuclease, 4.0. When the rates are corrected for the autoxidation rate, it is apparent that ribonuclease completely inactivated cytochrome oxidase.

*Cytochrome Systems, Spectrophotometric Technique*—The oxidation and reduction of cytochrome *c* was used as a basis for studying cytochrome oxidase, coenzyme I-cytochrome *c* reductase, and succinic dehydrogenase, following the principles and method of calculation described earlier in this series (14). All these enzymes are inactivated by ribonuclease. Dilute mouse liver homogenates were used as the source of enzyme and were filtered through bolting silk (Schindler Standard No. 20 obtained from the Allis-Chalmers Co.) to eliminate small shreds of connective tissue. The test is so sensitive that only 0.02 cc. of a 1 per cent homogenate is sufficient to reduce the cytochrome *c* in a period of several minutes. Since the dry weight of this amount of liver is only 60  $\gamma$ , the weight of active enzyme required must be very small. In order to study the effect of ribonuclease, tubes were made up with liver homogenate alone and with ribonuclease, with concentrations and incubation times determined from Figs. 1 and 2 and with water added to give a final liver concentration of 1 per cent. Fresh and incubated controls were used.

When cytochrome oxidase was to be studied, the solution of cytochrome in phosphate buffer was treated with one or two minute grains of solid  $\text{Na}_2\text{S}_2\text{O}_4$  which immediately reduced all of the cytochrome to the ferrous state. Following this treatment, the liver homogenate was added with a drawn-out pipette. The solution was mixed, and the zero time was noted. The solution was then transferred to an absorption cell, placed in the spectrophotometer and the first reading was taken exactly 30 seconds from the zero time. Values for  $I_0$  and  $I$  at 550  $\text{m}\mu$  were then taken at 15 second intervals until the reaction appeared to be definitely slowing down and the initial rate at which  $\log I_0/I$  changed was used to calculate enzyme activities. Fig 3 shows that cytochrome oxidase was inhibited by ribonuclease, in confirmation of the manometric experiments. Since the inactivation of cytochrome oxidase would provide adequate explanation for the results with succinoxidase, it was of particular interest to determine whether the succinic dehydrogenase was also inactive.

Succinic dehydrogenase was studied by measuring the rate of cytochrome *c* reduction in the presence of  $1/3,000$  cyanide, which was sufficient to prevent

completely the reoxidation of cytochrome *c* by cytochrome oxidase but was a low enough concentration to avoid appreciable combination with cytochrome *c* itself in the time covered (14). In these tests, the homogenate was added to the buffered cytochrome *c* solution and allowed about a minute in order to oxidize completely the cytochrome *c*. Cyanide was then added to block the oxidase, and the reaction catalyzed by succinic dehydrogenase was initiated by adding sodium succinate. Readings were then taken every 15 seconds in the spectrophotometer to obtain the data shown in Fig 3. Determinations of cytochrome oxidase and succinic dehydrogenase made on the same incubation mixtures showed that both enzymes were inactivated to the same extent by ribonuclease.

Cytochrome *c* can be reduced not only by succinic dehydrogenase but also by enzymes which transport electrons from the reduced coenzymes I and II. In order to distinguish between the two latter enzymes, which are specific for their respective substrates (5), we have called them coenzyme I-cytochrome *c* reductase and coenzyme II-cytochrome *c* reductase, following the nomenclature of Haas, Horecker, and Hogness (15) who applied the general name of cytochrome reductase to the enzyme which we prefer to call coenzyme II-cytochrome reductase. It appears that there is a family of cytochrome reductases and that succinic dehydrogenase may be considered as a member of this group, at least until it has been shown to consist of more than one component. In the present work, we studied the coenzyme I-cytochrome *c* reductase by using sodium dihydrocoenzyme I as a substrate (5). The tests were made in a manner identical with that used for succinic dehydrogenase, except that the reduced coenzyme was used in place of succinate. Experiments run parallel with succinic dehydrogenase are shown in Fig 4. Both enzymes were completely inactivated by ribonuclease in this experiment. According to the earlier ideas of the mechanism of hydrogen transport, the oxidation of  $\text{CoH}_2\text{I}$  was mediated by succinic dehydrogenase and, if this concept were correct, the inhibition of CoI-cytochrome *c* reductase by ribonuclease could be explained on the basis of the inhibition of succinic dehydrogenase. Although this concept was considerably weakened by the demonstration that the oxidation of a coenzyme I system could proceed under conditions in which succinate could not be oxidized (16), it seemed desirable to show that under the conditions employed in the experiment shown in Fig 4, the results achieved earlier in the over-all system could still be obtained. Therefore, simultaneous tests of the rate of reduction of cytochrome *c* by  $\text{CoH}_2\text{I}$  and by succinate in the presence of malonate were made. Fig 4 shows that, in the presence of sufficient malonate to block succinic dehydrogenase completely, the coenzyme I-cytochrome *c* reductase is completely unaffected. From this fact it may be concluded that the oxidation of coenzyme I is not mediated by the intermediate oxidation and reduction of the succinate-fumarate system and therefore that ribonuclease

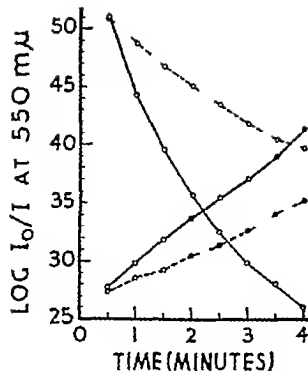


FIG 3

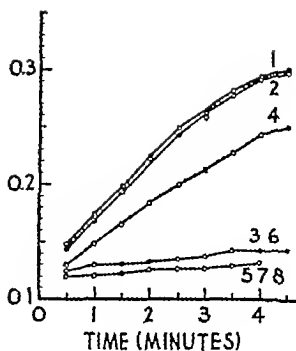


FIG 4

FIG 3 Inhibition of succinic dehydrogenase and cytochrome oxidase by ribonuclease. A 5 per cent mouse liver homogenate was incubated with an equal volume of 0.2 per cent ribonuclease for 90 minutes and then diluted with water to give a liver concentration of 1 per cent. Fresh and incubated controls were also diluted to 1 per cent. When cytochrome oxidase was tested, each absorption cell contained 1.0 ml. of sodium phosphate pH 7.4, 0.2 ml. of  $3.62 \times 10^{-4}$  M cytochrome *c*, and 1.78 ml. of water. After adding a grain or two of solid  $\text{Na}_2\text{S}_2\text{O}_4$  to reduce the cytochrome *c*, 0.02 ml. of 1 per cent liver homogenate was added, and readings were begun. When succinic dehydrogenase was measured, each cell contained phosphate and cytochrome as above, plus 1.58 ml. of water and 0.02 ml. of 1 per cent liver homogenate. The reaction was initiated by adding 0.2 ml. of a 1:1 mixture of 0.01 M  $\text{NaCN}$  and 0.5 M sodium succinate. In the chart, increases in  $E_{550}$  show succinic dehydrogenase activity and decreases in  $E_{550}$  show cytochrome oxidase activity. Incubated controls are shown in solid lines, and samples incubated with ribonuclease are shown in dotted lines. Fresh controls were not significantly different from incubated controls and are therefore not shown.

FIG 4 Inhibition of succinic dehydrogenase and coenzyme I-cytochrome reductase by ribonuclease. Inhibition of succinic dehydrogenase but not of coenzyme I-cytochrome *c* reductase by malonate. A 2 per cent mouse liver homogenate was diluted with water and ribonuclease solution to give a final concentration of 1 per cent liver and 0.067 per cent ribonuclease. Fresh controls yielded results identical with those of the incubated controls and therefore are not shown. Each absorption cell contained 2.5 ml. of 0.04 M sodium phosphate pH 7.4, 0.1 ml. of  $3.62 \times 10^{-4}$  M cytochrome *c*, and 0.1 ml. of 0.01 M  $\text{NaCN}$ , plus water to make a final volume of 3.0 ml. after all additions, which included various combinations of the following: liver homogenate, 0.02 ml.; 0.1 M malonate, 0.1 ml.; 0.5 M succinate, 0.1 ml.; and 0.005 M  $\text{CoH}_2\text{I}$ , 0.1 ml. The curves shown correspond to the following combinations: curve 1, liver +  $\text{CoH}_2\text{I}$ ; curve 2, liver +  $\text{Co}_2\text{HI}$  + malonate; curve 3, ribonuclease-treated liver +  $\text{CoH}_2\text{I}$ ; curve 4, liver + succinate; curve 5, liver + succinate + malonate; curve 6, ribonuclease-treated liver + succinate; curve 7,  $\text{CoH}_2\text{I}$  only; curve 8, liver only.

inactivates CoI-cytochrome *c* reductase *per se*. The data also show that cytochrome *c* is not the locus of ribonuclease action.

### *Non-Cytochrome Systems*

**Catalase**—The enzyme catalase resembles the cytochrome system insofar as it contains an iron porphyrin prosthetic group. Nevertheless, it appears to be completely unaffected by ribonuclease. A 5 per cent mouse liver homogenate was diluted to a final concentration of 0.02 per cent, and 0.4 ml of this solution (equivalent to 24γ of dry liver) was placed in the side arms of Warburg flasks. The main compartment contained 1.0 ml of 0.1 M sodium phosphate pH 7.4, 0.6 ml of H<sub>2</sub>O, and 1.0 ml of 0.075 per cent H<sub>2</sub>O<sub>2</sub> which was freshly prepared from Merck's superoxol. After equilibration at 38°, the side arm was tipped, and the evolution of oxygen was measured by taking readings at 2 minute intervals. In the first tests, the liver homogenate was incubated with ribonuclease under conditions comparable to those in Fig. 1, following which the mixture was diluted to give 0.02 per cent liver. Since this procedure might facilitate dissociation of any complex which might have been formed between catalase and ribonuclease, the final procedure was to incubate a mixture containing 0.02 per cent liver and 0.1 per cent ribonuclease, so that the ratio of the dry weights was 24γ of liver to 400γ of ribonuclease instead of 3,000γ of liver to 400γ of ribonuclease (which gave nearly complete inhibition in 2 hours in the case of succinoxidase as was shown in Fig. 1). In an experiment in which the liver was incubated for 2 hours with ribonuclease, using both the 5 per cent and 0.02 per cent homogenates and employing both fresh and incubated controls, the ribonuclease was found to exert no effect upon the catalase in either instance. The results, expressed in μl O<sub>2</sub> evolved in the first 10 minutes per 24γ of dry liver, were as follows: for the ratio 400γ ribonuclease/24γ liver: fresh controls, 103 and 99, incubated controls, 101 and 98, incubated with ribonuclease, 97 and 96, for the ratio 400γ ribonuclease/3,000γ liver: fresh controls, 117 and 128, incubated controls, 100 and 104, incubated with ribonuclease, 131 and 129. As a further control, a homogenate was incubated with ribonuclease, and aliquots were simultaneously tested for catalase and cytochrome oxidase. The cytochrome oxidase was completely inactivated, while the catalase was unaffected.

**Phosphatase**—Kabat has reported that the particulate matter in the cytoplasm of kidney cells contains an active alkaline phosphatase (7), one might expect this enzyme to be inhibited by ribonuclease, following the results with the succinoxidase system. However, a series of tests revealed no significant inhibition of the phosphatase by ribonuclease. The activity of the enzyme was measured in the following way: a 5 per cent kidney homogenate was incubated with varying amounts of ribonuclease and for varying time intervals. Concentrations of ribonuclease were used ranging from 500γ/25 mg of kidney

to 2,000 $\gamma$ /25 mg of kidney. The incubation times were 30, 60, 90, and 120 minutes. In some experiments the ribonuclease was incubated with kidney for as long as 6 hours. At the end of the incubation periods, 0.1 cc. samples of the incubation mix were removed and pipetted into tubes containing 0.9 ml.  $H_2O$ , 1.0 ml. of 0.1 M veronal buffer, pH 9.0, and 0.5 ml. of 0.03 M  $MgCl_2$ . At zero time, 0.5 ml. of 0.3 M sodium  $\beta$ -glycerophosphate was added, and the whole was incubated for time intervals ranging between 20 and 30 minutes at 37°. The reactions were stopped by the addition of 1.0 ml. of 20 per cent trichloroacetic acid which also precipitated the protein. 1 ml. aliquots were used for the determinations of inorganic phosphorus. The results of a typical experiment expressed as  $\gamma$  of inorganic phosphorus liberated in 20 minutes by 2.5 mg. of liver were as follows: fresh control, 77; incubated control, 88; incubated with ribonuclease, 77. The incubation time with the ribonuclease in the above experiment was 2 hours, the ribonuclease concentration was 950 $\gamma$ /25 mg. liver. In the same experiment, using the same controls and the same ribonuclease mix, succinoxidase and cytochrome oxidase assays were also carried out. The succinoxidase and cytochrome oxidase activities were inhibited completely.

Eight experiments were carried out in all, testing the action of ribonuclease on phosphatase. The average inhibition by ribonuclease for the whole group was 10 per cent.

*Adenosine Triphosphatase*—The experiments with alkaline phosphatase are subject to the criticism that the significance of this enzyme in the relation to the hydrogen transport mechanism is obscure. However, the enzyme adenosine triphosphatase is probably closely integrated with the respiratory enzymes since its substrate is synthesized by the esterification of inorganic phosphate using the energy of both glycolysis and respiration, and, in fact, the dehydrogenation of succinate has been related to phosphate esterification (17). The activity of the enzyme was measured on the basis of the rate of liberation of inorganic phosphate from pure adenosine triphosphate (ATP) by dilute liver homogenates.<sup>1</sup> Each flask contained 0.5 ml. of 0.1 M KCl-glycine buffer pH 9.1, 200 $\gamma$  of ATP in a volume of 0.4 ml., 0.45 ml. of 1 per cent liver homogenate, 1.35 ml. of water. After incubating for 45 minutes, 0.3 ml. of 100 per cent trichloroacetic acid was added, and inorganic phosphate was determined on the filtrate. The liver homogenates were incubated with ribonuclease as in Fig. 1 both fresh and incubated controls were used. The results, expressed in  $\gamma$  of inorganic phosphorus liberated per flask were as follows: fresh control, 25; incubated control, 23; incubated with ribonuclease, 22; ribonuclease alone, 0.6. As a further control, a homogenate was incubated with ribonuclease, and aliquots were simultaneously tested for succinic dehydrogenase and adenosine

<sup>1</sup> The assay technique is still being developed and will be reported in detail later.

triphosphatase The succinic dehydrogenase was completely inactivated while the adenosine triphosphatase was unaffected

*Urease*—A large number of diverse compounds have been shown to inhibit succinic dehydrogenase on the basis of reaction with an essential SH group on the enzyme (18) These compounds also inhibit urease by the same mechanism (13) Traces of copper ions and non-specific oxidants fall in this category, and it is apparent that if ribonuclease contained a few tenths of a per cent of such substances as an impurity, succinic dehydrogenase would be inactivated in much the same manner as shown in Fig 1 However, in such an event, urease would be inhibited in a similar fashion, and tests have shown that it is not The tests were carried out as follows the side arms contained 400 $\gamma$  of ribonuclease in 0.2 ml of water and 40 $\gamma$  of commercial urease in 0.2 ml water, the main flasks contained 1.0 ml of 0.1 M pyrophosphate buffer at pH 5.0 plus 0.3 ml of 2 M urea and water to give 3.0 ml after the urease and ribonuclease were added The ribonuclease was added to the flasks at various times as in the succinoxidase experiment described in Fig 1 and, at the end of the 2 hour period, the side arms were tipped and the CO<sub>2</sub> evolution was measured The results, expressed as  $Q_{CO_2}$  values, were as follows fresh control, 9,250, incubated control, 9,620, incubated with ribonuclease for 30 minutes, 8,800, for 60 minutes, 9,040, for 90 minutes, 8,840, and for 120 minutes, 8,690 None of these figures differs significantly from the mean, showing that ribonuclease does not inhibit urease From this fact, it may be inferred that the inhibition of succinic dehydrogenase is not due to a non-specific oxidation of sulfhydryl groups either by ribonuclease or by any impurity which might be present

*Xanthine Oxidase*—It has been suggested that succinic dehydrogenase may be a flavoprotein (19, 20), and it is almost certain that xanthine oxidase is a flavoprotein (21) It was, therefore, of interest to test the effect of ribonuclease on xanthine oxidase The tests were carried out as follows the side arms contained 120 $\gamma$  of partially purified xanthine oxidase in a volume of 0.1 ml plus 400 $\gamma$  of ribonuclease in a volume of 0.2 ml, the main flasks contained 1.0 ml of 0.1 M sodium phosphate pH 7.4, 0.2 ml of 0.8 per cent sodium xanthate, and water to make a final volume of 3.0 ml The ribonuclease and xanthine oxidase were added to the various flasks at the same times, and, after various periods of time, the side arms were tipped and the oxygen uptake was measured in flasks containing ribonuclease and in parallel control flasks The results, expressed in the basis of the oxygen uptake during the first 10 minutes, were as follows after 30, 60, 90, and 120 minute periods of incubation, respectively controls, 97, 94, 90, and 96, with ribonuclease, 101, 103, 97, and 101 It is apparent that the xanthine oxidase was unaffected by ribonuclease

#### DISCUSSION

The data presented above show that of the eight enzymes tested, ribonuclease specifically inhibits only CoI-cytochrome reductase, succinic dehydro-

genase, and cytochrome oxidase. On the basis of Loring's work, this fact may be taken as an indication that a ribonucleic acid is in some way associated with these enzymes. If this is indeed the explanation for the results, then the failure of ribonuclease to inhibit urease and xanthine oxidase is reasonable enough; these enzymes are highly purified and almost certainly do not contain any ribonucleic acid. But in the case of the alkaline phosphatase, there is evidence that the enzyme is attached to particles (7) and that these particles may be no different from those described by Claude (8). The present evidence would seem to indicate that the phosphatase is not associated with ribonucleic acid. The evidence obtained from negative ribonuclease experiments becomes inconclusive, however, when it is realized that all of the enzymes which we have found to be inhibited by ribonuclease are able to act *only when they are associated with cytochrome c*. Since ribonuclease does not act on cytochrome *c* directly, it is possible that succinic dehydrogenase, CoI-cytochrome *c* reductase, and cytochrome oxidase are components of a complex ribonucleoprotein of macromolecular dimensions and that, when ribonuclease acts on the ribonucleic acid contained therein, it prevents cytochrome *c* from approaching the enzyme components with the result that those enzymes whose action is dependent upon cytochrome *c* appear to be inactivated. Other enzymes like phosphatase and catalase could conceivably be in the same or in a similar complex but they do not require cytochrome *c* for their action, and molecules such as  $\beta$ -glycerophosphate and hydrogen peroxide which are relatively small compared with cytochrome *c* (molecular weight 13,000) might reach their activating centers unhindered. The CoII-cytochrome *c* reductase in a homogenate should also be inhibited by ribonuclease, but the CoII-cytochrome *c* reductase which has been isolated from yeast following autolysis (15) should not be affected by ribonuclease according to this concept.

The experiments on CoI-cytochrome *c* reductase seem to merit special attention, since the fact that this enzyme and succinic dehydrogenase appear to be closely associated might be taken as evidence in support of the Szent-Györgyi theory. However, the evidence is explained adequately on the basis of their rôle as cytochrome *c* reductases and implies nothing which can be used to prove that the oxidation of  $\text{CoH}_2\text{I}$  is mediated by the succinate-fumarate system. The experiments with malonate, shown in Fig. 4, indicate quite strongly that the activating center for succinate is distinct from that which activates  $\text{CoH}_2\text{I}$  and provide striking confirmation for the earlier demonstration of this fact (16). This is the third experimental demonstration of this point, the second was provided by Straub (22) of the Szent-Györgyi school. Straub was able to inactivate the succinic dehydrogenase contained in a muscle pulp by keeping it at pH 9 for an hour, then neutralizing back to pH 7.3. The resulting preparation was completely unable to oxidize succinate but showed scarcely any loss in the ability to oxidize reduced CoI (malate system). As a result of this experiment Straub stated that reduced flavoprotein (which we identify



as CoI-cytochrome *c* reductase) "does not react with succinic dehydrogenase but has its direct connection with the oxidizing system" This statement is much more far-reaching than that of Potter (16, 23) whose experiments with malonate, which were analogous to Straub's experiments with alkaline incubation, led to the conclusion that there is at least a path alternate to the Szent-Gyorgyi mechanism Straub's statement appears to renounce the Szent-Gyorgyi mechanism completely The other data pertinent to the Szent-Gyorgyi theory have been previously discussed (24), and there now seems to be no reason for its continued application in the absence of new supporting evidence

#### SUMMARY

1 The effect of ribonuclease on various enzyme systems was studied as one approach to the problem of whether or not these enzymes are contained in macromolecules of ribonucleoprotein nature in protoplasm

2 Ribonuclease inhibited CoI-cytochrome *c* reductase, succinic dehydrogenase, and cytochrome oxidase, all of which require cytochrome *c* in order to function Ribonuclease did not act on cytochrome *c*

3 Ribonuclease did not inhibit urease, xanthine oxidase, catalase, alkaline phosphatase, or adenosine triphosphatase under the conditions employed

4 It was suggested that ribonuclease acted sterically by preventing contact between cytochrome *c* and its activating centers

5 It was suggested that the enzymes inhibited may be contained in a ribonucleoprotein of macromolecular dimensions but that the enzymes not inhibited are not necessarily excluded from such a complex by the data presented

6 Further evidence against the Szent-Gyorgyi theory of hydrogen transport was presented and discussed

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# PACEMAKERS IN NITELLA

## III ELECTRICAL ALTERNANS

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Alternans is a convenient term used to denote the alternation of strong and weak heart beats. There may be an alternation in the electrical responses which accompany the contractions.<sup>1</sup>

Alternation in electrical responses is also found<sup>2</sup> in *Nitella*, as seen in Fig. 1.

The records were made by leading off from three regions *C*, *D*, and *E* on the *Nitella* cell to a fourth spot *F* as described in previous papers,<sup>3</sup> and recording each lead by means of an Einthoven galvanometer with 3 strings.

In no case was electrical stimulation applied. The impulses came from regions of the cell which acted as pacemakers. Such pacemakers can sometimes be produced artificially by placing a drop of 0.01 M KCl in contact with the cell. This holds the P.D. constant approximately at zero; a neighboring spot can discharge into this and as soon as its P.D. builds up in the process of recovery it can discharge again and so set up a long train of action currents. In most cases such a drop was applied at the right hand end of the cell but in many instances the pacemaker proved to be elsewhere.

The cells were kept in 0.01 M NaCl before the record was made; this tends to promote quick action currents.<sup>4</sup>

In the record shown in Fig. 1 the impulse passed along the cell from *D* to *C* (as is evident in other parts of the record not shown here), giving a full response at *D* but only a partial response at *C*. (The record at *E* is not shown.)

<sup>1</sup>White P. D. Heart disease. New York: The Macmillan Company, 2nd edition, 1937, 569.

<sup>2</sup>Osterhout, W. J. V. and Hill S. E. *J. Gen. Physiol.* 1938-39, **22**, 115.

<sup>3</sup>The cells, after being freed from neighboring cells, stood in the laboratory at  $15^{\circ} \pm 1^{\circ}\text{C}$  in Solution A (cf. Osterhout W. J. V. and Hill S. E. *J. Gen. Physiol.* 1933-34, **17**, 87) for several days. They belonged to Lot B (cf. Hill, S. E. and Osterhout W. J. V. *Proc. Nat. Acad. Sci.*, 1938, **24**, 312).

The measurements were made on *Nitella flexilis* Ag. using the technique described in former papers (Hill S. E. and Osterhout W. J. V. *J. Gen. Physiol.* 1937-38, **21**, 541; Osterhout W. J. V. and Hill S. E. *J. Gen. Physiol.* 1933-34, **17**, 87; 1934-35, **18**, 499; 1938-39, **22**, 115). Records were made at about  $22^{\circ}\text{C}$ .

The distance between *C* and *D* and between *D* and *E* was 25 mm. and that between *E* and *F* 37 mm. unless otherwise stated.

There was no indication of injury in these experiments.

<sup>4</sup>Hill S. E. and Osterhout, W. J. V. *J. Gen. Physiol.* 1938-39, **22**, 91.



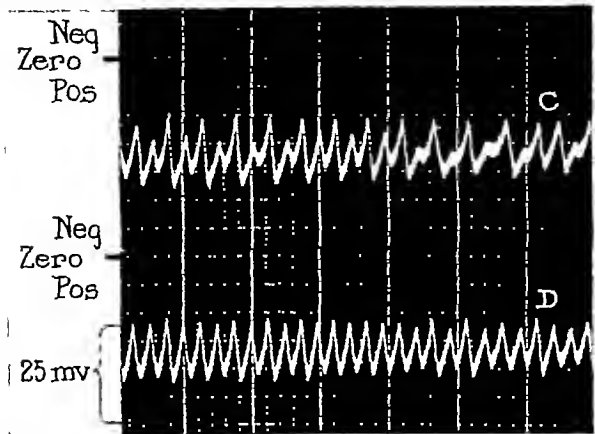


FIG 2 The impulse passes from *D* to *C* Every other impulse produces only a partial response at *C* and this is gradually reduced to a mere hump on the upstroke

*C* and *D* are in contact with 0.005 M  $\text{CaCl}_2$  + 0.001 M  $\text{KCl}$  and *F* is in contact with 0.01 M  $\text{KCl}$  which keeps the P.D. constant approximately at zero

The cell was freed from neighboring cells and kept for 30 days at  $15 \pm 1^\circ\text{C}$  in Solution A and then placed for 45 minutes in 0.01 M  $\text{NaCl}$  at about  $22^\circ\text{C}$  before the record was made.

Heavy time marks 5 seconds apart.

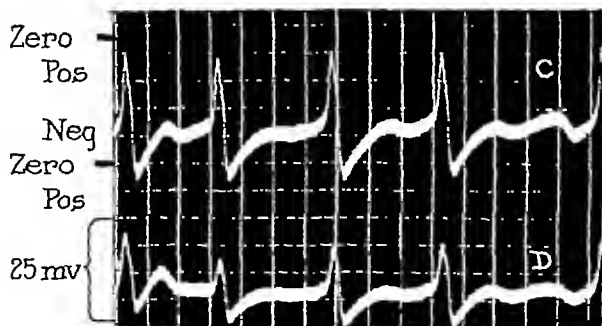


FIG 3 The impulse passes from *C* to *D* The partial response is variable and is reduced in places to a very slight hump

*C* and *D* are in contact with 0.01 M  $\text{NaCl}$  and *F* is in contact with 0.01 M  $\text{KCl}$  which keeps the P.D. constant approximately at zero.

The cell was freed from neighboring cells and kept for 30 days at  $15 \pm 1^\circ\text{C}$  in Solution A and then placed for 45 minutes in 0.01 M  $\text{NaCl}$  at about  $22^\circ\text{C}$  before the record was made.

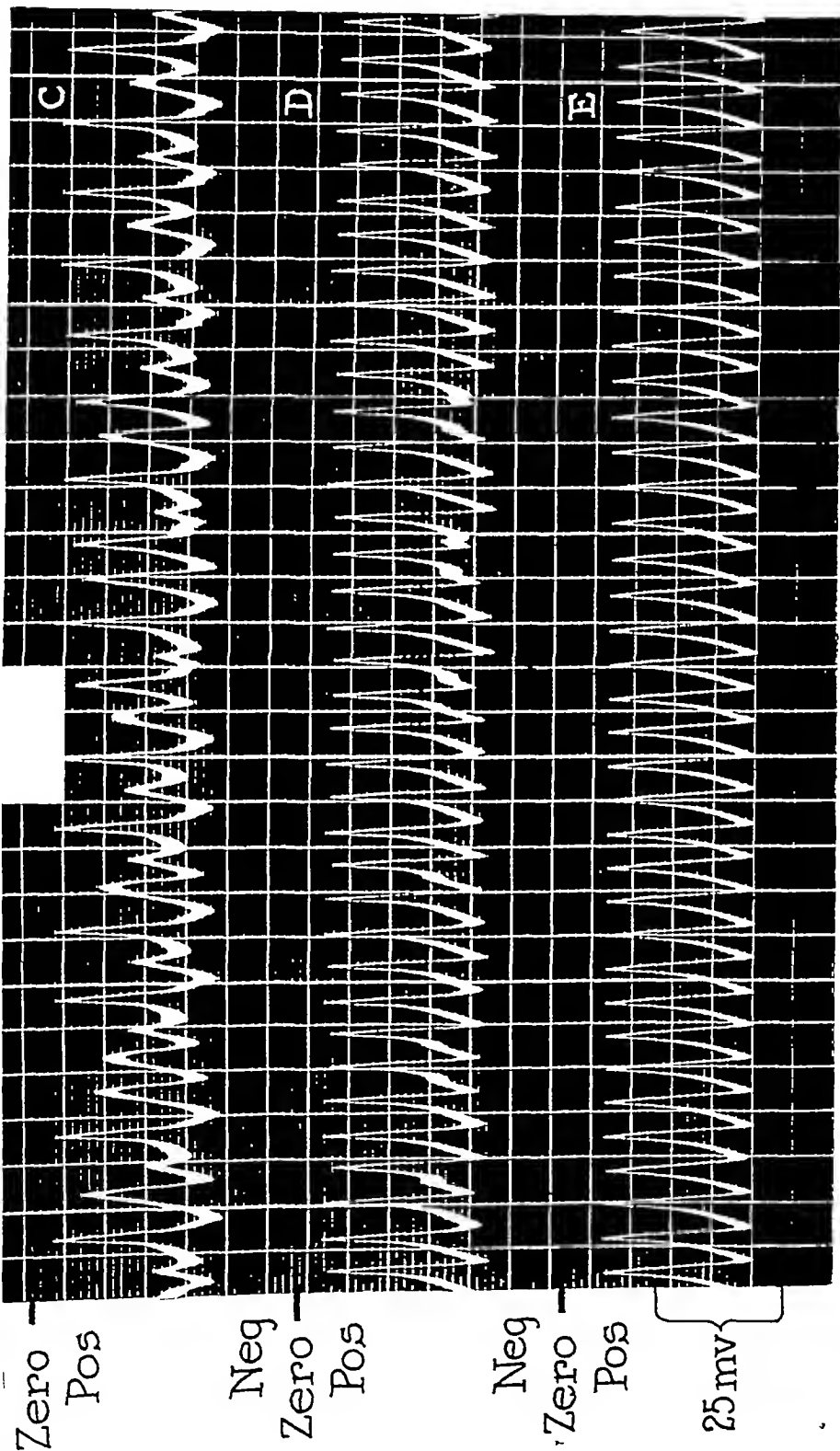


FIG. 4 The impulse passes from E to C and in several cases on reaching D produces an abnormal response showing a small hump or thickening on the upstroke. At C partial responses are observed. The timing shows that the humps at D do not represent separate impulses as they do in Figs 2 and 3.

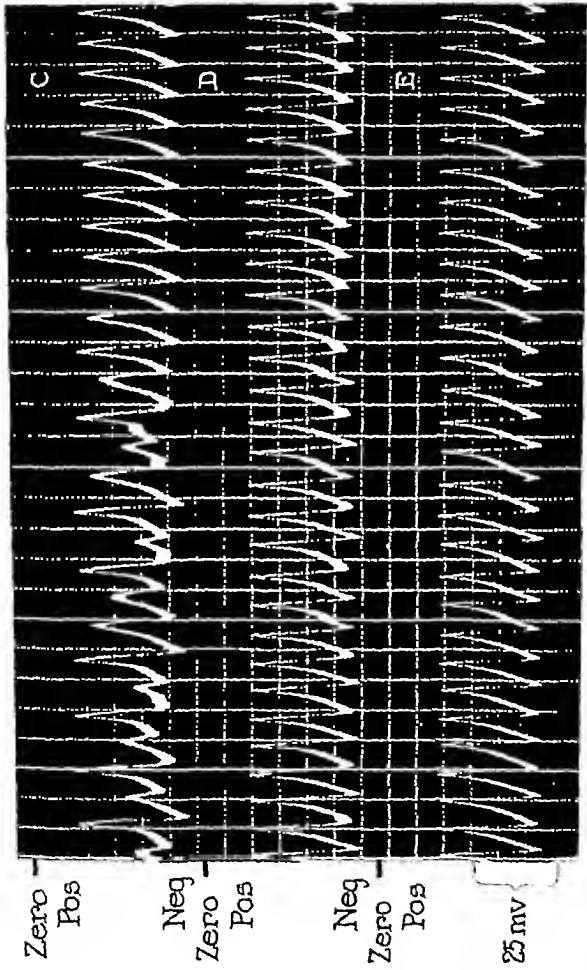


FIG. 5 Shows that a partial response can change to a normal one. The record was continued for 6 minutes longer with no reappearance of a deficient response at 19. The impulse passes from D to C. In certain cases there is a slight hump at D.



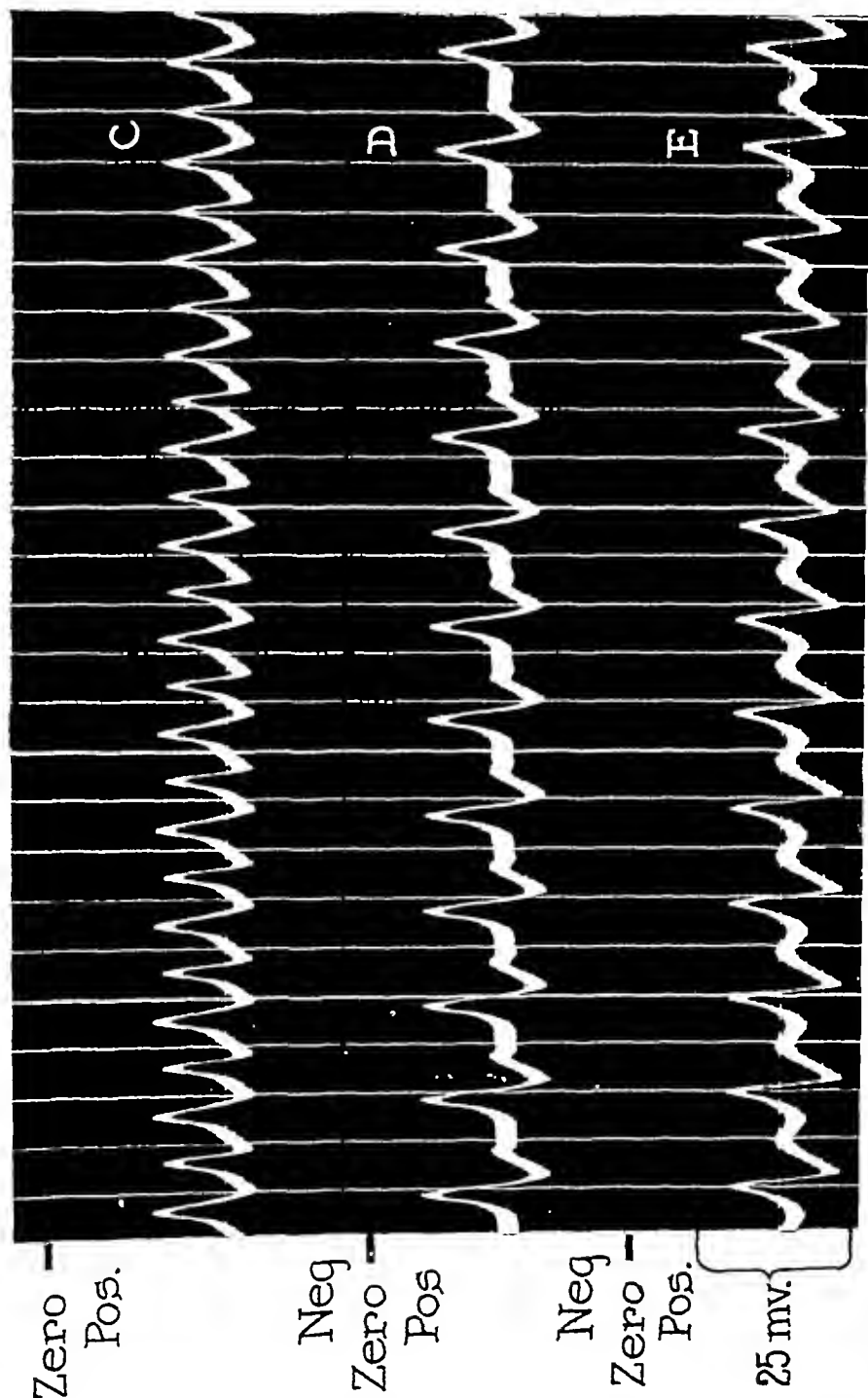


FIG. 6. The impulse passes from C to D and E. Alternans appears at D and E. The record at D might give the impression of a base line denoting complete recovery below which the curve dips to give "after positivity" such an impression would be misleading as is evident by comparison with other figures in this paper. C and E are in contact with 0.005 M  $\text{CaCl}_2$ . D is in contact with 0.01 M  $\text{NaCl}$  and E in contact with 0.01 M  $\text{KCl}$  which I assume

represents a 'base line' indicating the resting potential and that the part of the curve which dips below this represents after positivity. This is evidently not the case.<sup>3</sup>

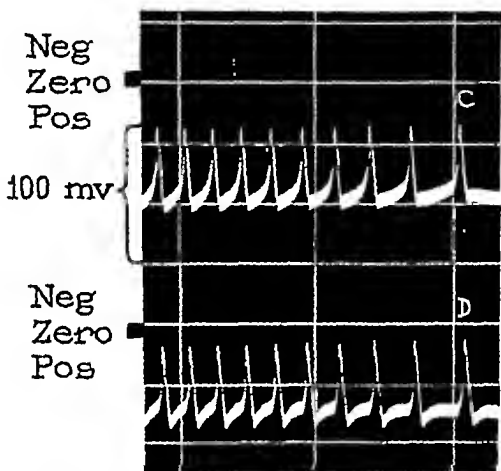


FIG. 7. The impulse passes from C to D. Both spots show thickenings of the upstroke which are similar but not identical.

C and D are in contact with 0.01 M NaCl and F is in contact with 0.01 M KCl which keeps the P.D. constant approximately at zero. The leads are 18 mm apart.

The cell was freed from neighboring cells and kept for 40 days in Solution A at  $15 \pm 1^\circ\text{C}$ . It was placed for 2.5 hours in 0.01 M NaCl at about  $22^\circ\text{C}$  before the record was made.

Heavy time marks 5 seconds apart.

We also meet with curves as in Fig. 7 where the thickening is not in the middle of the upstroke but at the bottom and there is no hump here it does not seem probable that the thickening represents alternans any more than the somewhat similar thickening at D in Fig. 5. Whether a thickening or even a slight hump signifies a deficiency in any given case must be decided by the timing relations.

Why does a partial response occur? Possibly because the approaching

<sup>3</sup> Cf. Hill S. E. and Osterhout W. J. V. *J. Gen. Physiol.* 1938-39 22, 103 (Fig. 14).

impulse finds the protoplasm in a "relatively refractory state" which follows stimulation. By the time the next impulse arrives the protoplasm may have passed out of this state so that it is able to give the full response.

In what is here called a full response the magnitude may be much less than normal and this may be due to the relatively high frequency. Since the frequency is at least 30 times the normal it is quite possible that each action curve

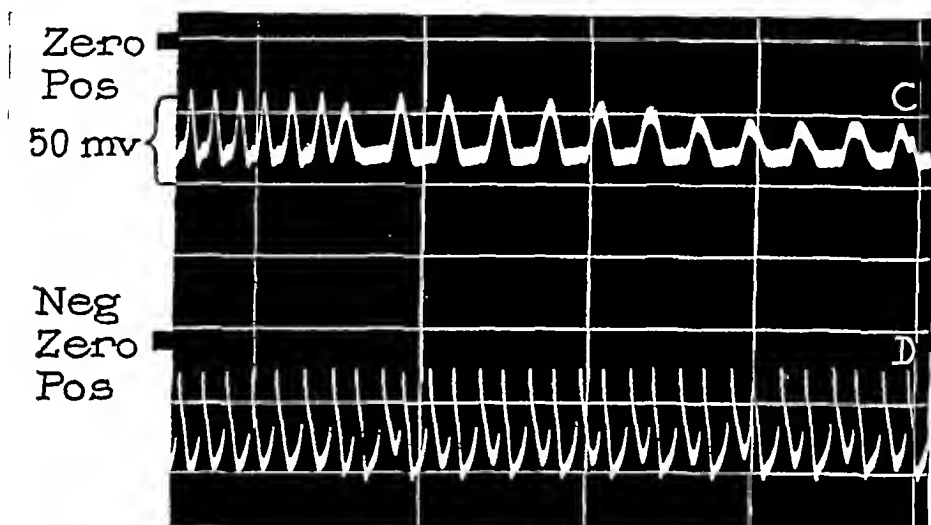


FIG 8 Shows the sudden appearance of a 2 to 1 ratio. The impulse passes from *D* to *C*. The first 7 action currents at *D* are followed by corresponding action currents at *C* but after this every other action current at *D* is followed by one at *C*; this is presumably because every other impulse finds *C* in the absolutely refractory state and is therefore unable to produce any response. (Recovery at *D* is incomplete in some cases.)

*C* and *D* are in contact with 0.01 M NaCl and *F* is in contact with 0.01 M KCl which keeps the P.D. constant approximately at zero. The leads are 28 mm apart.

The cell was freed from neighboring cells and kept for 40 days in Solution A at  $15 \pm 1^\circ\text{C}$ . It was placed in 0.01 M NaCl for 3 hours at about  $25^\circ\text{C}$  before the record was made.

Heavy time marks 5 seconds apart.

in the record at *D* in Fig. 2, for example, is initiated in the relatively refractory state and is consequently of less than normal magnitude.

It is interesting to compare the deficient responses in *Nitella* with those observed in nerve. When the frequency exceeds a certain value in nerve the responses may at first be normal or nearly so but deficiencies in response may appear and become more pronounced as time goes on. The magnitude of response may fall off and regular alternans may make its appearance.<sup>6</sup>

<sup>6</sup> Cf. Gasser, H. S., The excitability cycle, in Erlanger, J., and Gasser, H. S., Electrical signs of nervous activity, The Eldridge Reeves Johnson Foundation for Medical Physics Lectures, Philadelphia, University of Pennsylvania Press, 1937, 173.

If the action curve is correlated with a movement of potassium, as elsewhere suggested,<sup>7</sup> we may say that the falling off in the magnitude of the response denotes a falling off in the magnitude of the movement of potassium which may perhaps be due to a change in permeability.

In some cases the protoplasm may be in the "absolutely refractory state" so that no response occurs. In this case<sup>8</sup> we may obtain a 2 to 1 ratio of responses,<sup>2</sup> as seen in Fig. 8. The absolutely refractory state in *Nitella* is extremely variable and may last from a few seconds to several minutes, depending on the nature of the cells, the time of year, and the treatment received in the laboratory.

In addition to the action curves shown here a variety of others are observed, many of which seem to be capable of explanation on the basis here set forth.

It is evident that regions of the cell not widely separated may react differently.<sup>3</sup> This requires further study.

#### SUMMARY

An electrical impulse traveling along a *Nitella* cell may produce a complete or a partial response. The two kinds of response may occur in regular alternation.

The partial response varies greatly and may be so far reduced as to appear as a local thickening in the upstroke of the action curve, usually accompanied by a more or less pronounced hump. In consequence a considerable variety of action curves is produced.

The observations show that different regions of the cell may react differently.

<sup>7</sup> Osterhout, W J V. *J. Gen. Physiol.* 1934-35 18, 215.

<sup>8</sup> In most cases each deficiency at C is preceded by a partial deficiency at D since the impulse arrives at D before recovery is complete.

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# THE EFFECT OF LOW OXYGEN TENSION ON TISSUE METABOLISM (RETINA)

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(Received for publication February 27, 1943)

## I

These experiments were undertaken initially to determine whether confirmation could be found for the observation of Laser (1937*a*) that in an atmosphere containing only 5 volumes per cent of oxygen, both respiration and glycolysis of rat retina were at or near their maximum rates. On the basis of his experiments with retina and other tissues Laser stated that "the Pasteur effect under the conditions of the experiments depends upon the oxygen tension rather than upon the rate of respiration." Thus the implications of his observation are such that it is important that they be either supported or challenged. Stern and Melnick (1941) repeated the experiment of Laser while determining the physiological absorption spectrum of the Pasteur enzyme but the data were obtained at a temperature of 27°. For this reason it was desirable to have further data at 38°.

Since manometric methods alone were employed by Laser, his work provided data for glycolysis in bicarbonate medium but not in phosphate medium. The nature of the buffer has been found to influence profoundly the metabolism of retina, particularly with respect to its magnitude and to its sensitivity to cyanide and carbon monoxide (Laser, 1936, 1937*a*, and 1937*b*). Therefore it was also of interest to test chemically whether the substitution of phosphate for bicarbonate buffer would affect the relationship between glycolysis and oxygen tension.

## II

The retinas obtained from three or six mongrel white rats were prepared in the light at room temperature, cut in half, and pooled. 5 minutes per rat were required for preparation. The retinas were suspended in a medium that contained NaCl 0.118 M, KCl 0.0024 M, CaCl<sub>2</sub> 0.0017 M, MgCl<sub>2</sub> 0.0007 M and glucose 0.011 M in addition to the buffer. In medium containing phosphate buffer (NaH<sub>2</sub>PO<sub>4</sub> 0.003 M and Na<sub>2</sub>HPO<sub>4</sub> 0.017 M) oxygen uptake was determined by the first method of Warburg (1923). The lactic acid that accumulated in the vessel during the preliminary period of equilibration and the experimental period was determined colorimetrically by the

\* Aided by a grant from the Milton Fund of Harvard University

method of Barker and Summerson (1941) In nine tests the lactic acid formed in the equilibration period at different oxygen tensions was  $25 \pm 1.8$  per cent of the total found at the end of a 2 hour experiment Accordingly, 75 per cent of this total was used in calculating the  $Q_G$   $Q_G = 0.004$  mg of lactic acid, per milligram of tissue per hour In medium containing bicarbonate buffer ( $\text{NaHCO}_3$ , 0.024 M and 5 per cent  $\text{CO}_2$ ), both oxygen uptake and lactic acid output were determined by the second method of Warburg (1924) In this method the estimation of lactic acid output includes the assumption that the respiratory quotient is 1 Actually the R Q of retina is lower (Laser, 1937a, Greig, Munro, and Elliott, 1939), but this fact does not impair the usefulness of the method

The gas mixtures were made by displacement of saturated  $\text{CaCl}_2$  solution from 20 liter carboys They were analyzed with the Haldane apparatus The mixtures were reported on a dry basis, it being understood that the water vapor at  $38^\circ$ , the temperature of the experiments, amounts to 7 volumes per cent Consequently the tension in atmospheres is equal to 0.93 times the volume per cent as stated in the body of the paper Laser's procedure was followed particularly with regard to the rate of shaking (160 cycles per minute) and, in bicarbonate medium, to preliminary equilibration of the vessels with the gas mixtures before adding the tissues At the conclusion of a 1 or 2 hour experimental period, the retinas were rinsed and dried to constant weight When lactic acid was to be determined chemically, the experiment was conducted for 2 hours to obtain a larger amount for analysis

### III

The data are summarized and compared with those of Laser in Table I In phosphate medium the only significant change in  $Q_{O_2}$  with time was an increase from the 1st to the 2nd hour with 0.5 per cent oxygen, in bicarbonate medium, the metabolism often fell off during the 2nd hour

The data for bicarbonate medium are in essential agreement with those of Laser Therefore not so many bicarbonate buffer experiments were done as with phosphate, where we differ with him In bicarbonate medium there was no significant decrease in oxygen uptake with oxygen mixtures between 95 per cent and 5 per cent, while at 5 per cent the lactic acid output was almost at the anaerobic level

In phosphate medium, oxygen uptake was more sensitive to oxygen tension Laser reported no change in oxygen uptake when the oxygen tension was reduced from 100 per cent to 5 per cent, whereas in our experience, oxygen uptake was 38 per cent less at 10 per cent oxygen than at 100 per cent, and 51 per cent less at 5 per cent oxygen The  $Q_{O_2}$  in 100 per cent oxygen, however, agreed with his figure of 17 When the individual data for respiration at different oxygen tensions were plotted against the corresponding data for glycolysis in Fig 1, a linear relationship was suggested This is in marked contrast to the situation in bicarbonate medium, where respiration and glycolysis varied with oxygen tension independently of each other

## IV

Burk (1939) has discussed and restated three main theories to explain the observed fact that in many kinds of living cells less alcohol or lactic acid accumulates in the presence of air than when oxygen is excluded. In brief they

TABLE I  
*Oxygen Uptake and Lactic Acid Output of Retina*

In bicarbonate medium the data are for the 1st hour only. In phosphate medium the data represent the average for 2 hours, in our experiments.

Oxygen tension	Laser's (1936, 1937a) data for retina		The present data with standard errors of the means.		
Vol. per cent	$Q_{O_2}$	$Q_G$	No. of observations	$Q_{O_2}$	$Q_G$
Bicarbonate medium					
95	30 32	45.0	5	$34 \pm 4.6$	$41 \pm 0.9$
10-12	29.2 35.0	68.1 70.8	—	—	—
3-6	27.85 29.0	71.0 67.1	6	$27 \pm 3.9$	$72 \pm 2.9$
0-0.5	—	88	3	$3 \pm 0.4$	$77 \pm 4.3$
Phosphate medium					
100	17.5	—	10	$17.4 \pm 1.0$	$20 \pm 1.7$
10-12	17.5	—	7	$10.8 \pm 0.7$	$26 \pm 2.4$
3-6	19.75 17.90	—	7	$8.5 \pm 1.1$	$37 \pm 1.6$
0.5	—	—	10	$1.3 \pm 0.4$	$44 \pm 2.3$

are first, "the fermentation intermediates are oxidized (Pasteur, Pfeffer, Pflüger)," second, "the fermentation intermediates are mainly resynthesized (Meyerhof)," and third, "the fermentation intermediates are mainly not formed" because "oxygen presumably maintains some reduced component of the fermentation enzyme system in an oxidized inactive form" (Lipmann, 1942). The results of Laser in bicarbonate medium, which our experiments confirm, support the third theory for they show that the rate of glycolysis



depended in these circumstances on the oxygen tension and not on the rate of respiration

A marked increase in the sensitivity of oxygen consumption to low oxygen tension was observed when phosphate medium was substituted for bicarbonate. The extent to which this change can be accounted for on the basis of a specific influence of the buffer on diffusion, is not known.

In connection with his studies on retina and other tissues, Laser (1937*a*) called attention to the fact that a 5 per cent oxygen mixture is nearer to the physiological oxygen tension of the tissues than is 100 per cent oxygen, com-

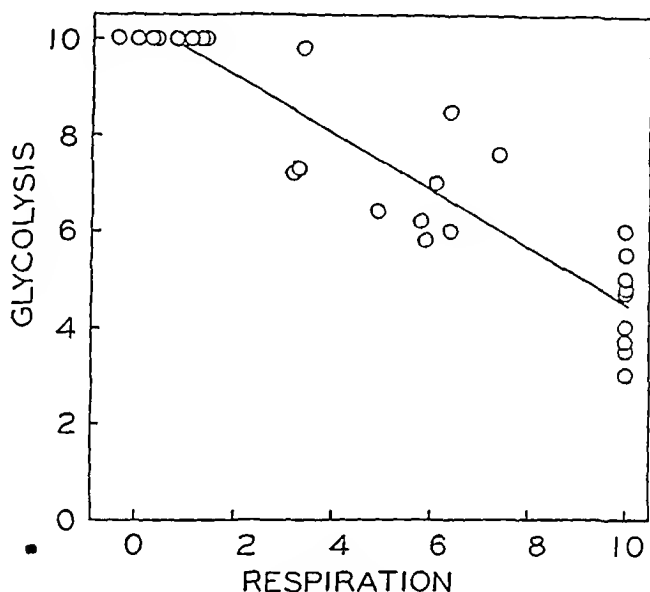


FIG 1 Oxygen uptake at different oxygen tensions as a fraction of the rate in 100 per cent oxygen in each experiment plotted against the corresponding value for lactic acid output as a fraction of the rate in 0.5 per cent oxygen

monly employed in experiments on surviving tissue. He believed also, with Warburg, that tissue metabolism was largely independent of oxygen tension. Since our data on oxygen consumption in phosphate medium, in contrast to Laser's results, show marked sensitivity to oxygen tension, it may be well to mention other instances of sensitivity to oxygen tension. The ingenious experiment of Laser (1932) with inflated rat lungs supported in the gas phase of the Warburg flask, should be recalled. Here, in a situation in which questions of diffusion and buffer specificity do not arise, the  $Q_{O_2}$  decreased from 7.1 to 4.6 when the oxygen tension changed from 100 per cent to 10 per cent. More recently, Kempner (1939) has criticized the Warburg point of view, and since then oxygen uptake-oxygen tension curves with critical oxygen tensions well above 5 per cent have been published for bone marrow (Warren, 1942), kidney

(Laser, 1942), and brain cortex, medulla, and spinal cord (Craig and Beecher, 1943). Of these, bone marrow and brain cortex exhibit also the linear relation between respiration and glycolysis just described for retina in phosphate buffer. One implication of this relation is that oxygen tension may act on the two processes through the mediation of a common agent.

#### SUMMARY

Lactic acid production by rat retina in a medium containing phosphate was studied chemically. One half as much lactic acid was found as in a medium containing bicarbonate. In our experience the rate of respiration in a phosphate medium was sensitive to oxygen tension, for it was 38 per cent lower at 10 per cent and 51 per cent lower at 5 per cent oxygen than at 100 per cent oxygen. Previously Laser had reported no decrease in respiration at 5 per cent oxygen in phosphate medium. In phosphate medium, when the oxygen tension was varied, respiration and glycolysis bore a reciprocal relationship to each other.

In bicarbonate medium, when the oxygen tension was lowered from 95 per cent to 5 per cent there was no significant change in the respiration, but glycolysis was increased nearly to the anaerobic level. This agrees with the earlier experiment of Laser in bicarbonate medium and adds support to his conclusion that the rate of glycolysis is controlled by oxygen tension rather than by the rate of respiration, under the conditions of the experiment.

We are indebted to Dr. Fritz Lipmann for many helpful suggestions during the course of this study and to Miss Anna Murphy for her careful assistance with the chemical determinations.

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# THE EFFECT OF CARBON DIOXIDE TENSION ON TISSUE METABOLISM (RETINA)

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## I

During the study of the effect of oxygen tension on the metabolism of retina (Craig and Beecher 1943), our attention was drawn to the observation of Laser (1936, 1937) that the metabolism of this tissue in medium containing bicarbonate is twice as great as it is in medium containing phosphate buffer. The purpose of the experiments described here was to investigate whether this quantitative difference might be related to the concentration of the carbon dioxide-bicarbonate system. Warburg, Posener, and Negelein (1924) had already demonstrated the sensitivity of anaerobic glycolysis in a tumor to the concentration of the carbon dioxide-bicarbonate buffer system.

## II

The material and its preparation, the media except for changes as noted and the methods were described in the preceding paper. The study of carbon dioxide tension presented two complications. In order to keep the pH constant it was necessary to vary bicarbonate in proportion to carbon dioxide. In order to maintain the osmotic pressure when bicarbonate was changed it was necessary to vary chloride inversely with bicarbonate. To minimize the alteration in the chloride concentration, one series was run at a lower pH.

## III

From the results on rat retina in Table I, the following are apparent. The chloride and pH changes did not alter the effect on metabolism of changes in carbon dioxide-bicarbonate concentration. Lowering the pH from 7.48 to 7.18 (as calculated from the Henderson-Hasselbalch equation) depressed glycolysis but had no significant effect on oxygen uptake. In both series the oxygen uptake was maximal at 5 per cent carbon dioxide, being significantly less at carbon dioxide tensions of 1 per cent and 20 per cent. In both series there was an increase in lactic acid output between 1 per cent and 5 per cent carbon dioxide but no further change beyond 5 per cent. The effects of carbon dioxide tension at pH 7.48 are shown in Fig. 1.

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TABLE I

*Effect of Carbon Dioxide Tension in Bicarbonate Medium on the Oxygen Uptake and Lactic Acid Output of Rat Retina*

Results for the 1st hour and standard error of the mean In recording the gas mixtures the vapor tension of water was not corrected for This amounts to 7 volumes per cent at 38°

CO <sub>2</sub> vol per cent	O <sub>2</sub> vol per cent	NaHCO <sub>3</sub> M × 1000	NaCl M × 1000	No of ob servations	Q <sub>O<sub>2</sub></sub>	Q <sub>G</sub>
Calculated pH 7.48						
1	99	5	157	16	16 ± 2.0	26 ± 1.2
5	95	24	138	10	31 ± 2.3	46 ± 2.0
10	90	48	114	4	29 ± 3.9	46 ± 1.9
20	80	96	66	5	19 ± 4.2	48 ± 3.3
Calculated pH 7.18						
1	99	2.5	165	4	15 ± 2.7	17 ± 1.8
5	95	12	155	4	25 ± 2.4	31 ± 5.7
20	80	48	119	4	15 ± 4.2	30 ± 5.1

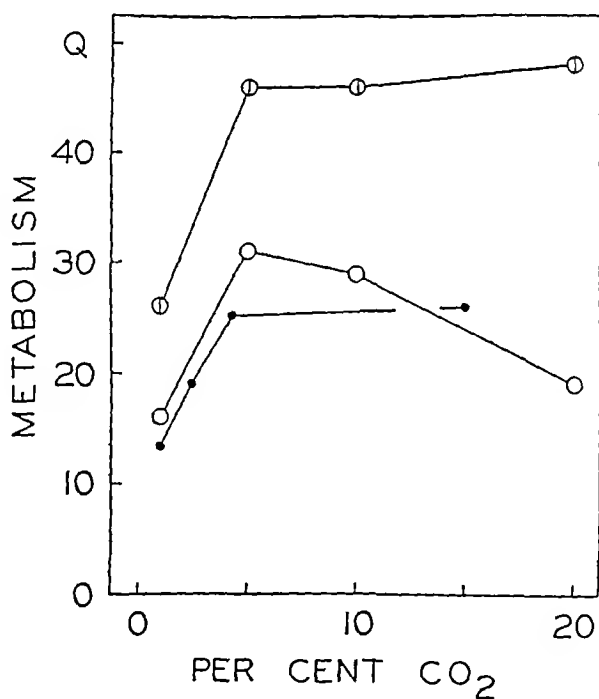


FIG 1 Barred circles refer to  $Q_G^{O_2}$  of retina (Table I), open circles to  $Q_{O_2}$  of retina (Table I), and closed circles to  $Q_G^{N_2}$  of sarcoma (Warburg, Posener, and Negelein, 1924)

From the data in Table II it will be seen that the rates of oxygen uptake and of lactic acid output in phosphate medium are of the same order as those in bicarbonate medium with 1 per cent carbon dioxide.

Recent indications that carbon dioxide takes part in the Krebs cycle in minced pigeon liver suggested the test of whether the effect of carbon dioxide tension when increased from 1 per cent to 5 per cent, could be duplicated by the addition of succinate. The results obtained when neutralized sodium

TABLE II

*Effect of 0.02 M Succinate on Oxygen Uptake and Lactic Acid Output*

Glucose was always present. Results as averaged over an experimental period of 2 hours, and standard errors of the means.

A. Phosphate medium

Succinate	No. of observations	$Q_{O_2}$	$\Sigma Q$	$Q_G$ Determined chemically	
				NaOH in inset	NaOH absent
Absent	6	$16.4 \pm 0.6$	$0.72 \pm 0.05$	$17 \pm 1.8$	$17 \pm 0.8$
Present	6	$18.4 \pm 1.2$	$0.69 \pm 0.05$	$20 \pm 1.4$	$22 \pm 1.6$

B. Bicarbonate medium with 1 per cent  $CO_2$  and 0.005 M  $NaHCO_3$ .

Succinate	No. of observations	$Q_{O_2}$	$Q_A$	$Q_G$	$Q_{Gl}$	$Q_G$
		Determined manometrically				Determined chemically
Absent	7	$12 \pm 2.1$	$31 \pm 2.9$	$19 \pm 1.8$	—	$14 \pm 1.3$
Present	7	$26 \pm 2.1$	$42 \pm 2.6$	$30 \pm 3.0$	$16 \pm 1.1$	$14 \pm 0.5$

\*  $Q_G = Q_A - Q_{O_2}$  in absence of succinate. Assumption the  $\Sigma Q$  in the absence of succinate is 1 but the extra oxygen uptake with succinate is not accompanied by carbon dioxide production.

†  $Q_G = Q_A - Q_{O_2}$  with succinate. Assumption the over-all  $\Sigma Q$  is 1.

succinate was made available to the retina in high concentration (0.02 M) in addition to glucose, are recorded in Table II.

In phosphate medium succinate had no significant effect on oxygen uptake, respiratory quotient or lactic acid output. In its inability to bring about the rapid oxidation of succinate in phosphate medium, rat retina differed from many normal tissues and resembled certain tumors (Craig Bassett and Salter, 1941). The slight variation in carbon dioxide tension resulting from the presence or absence of NaOH in the inset did not influence the lactic acid output in the paired vessels used for the determinations of  $\Sigma Q$  with phosphate medium. This point has been discussed by Laser (1942).

In bicarbonate medium with 1 per cent carbon dioxide, the control

were lower than the data for respiration and glycolysis given in Table I because they represented the mean of a 2 hour period in which the rates were declining. Succinate raised the  $Q_{O_2}$  to the level previously observed with 5 per cent carbon dioxide (Table I). It also raised  $Q_A$ , the sum of lactic acid production ( $Q_G$ ) and respiratory carbon dioxide. Since an independent chemical determination showed that lactic acid production was not altered by succinate, the increment in  $Q_A$  with succinate may be attributed to an increase in respiratory carbon dioxide output. Hence it is unlikely that the extra oxygen uptake due to succinate is the result of succinate dehydrogenation alone. Whether the substrate of the extra oxygen uptake was glucose or succinate remains to be determined.

#### IV

Sensitivity of lactic acid output by a tumor to carbon dioxide-bicarbonate concentration has been described by Warburg *et al* (1924). The data are shown in Fig. 1. Laser (1936, 1937) noted that the respiration of retina and of sarcoma 189 was lower in phosphate than in bicarbonate medium. Our results in the previous paper (Craig and Beecher, 1943) indicated that in retina, lactic acid production as well as oxygen uptake, was lower in phosphate than in bicarbonate, and lower by the same fraction. Although at 99 to 100 per cent oxygen, metabolism was quantitatively almost the same in phosphate medium and in bicarbonate medium with 1 per cent carbon dioxide, the possibility of a specific effect of phosphate has not been eliminated. The sensitivity of respiration in 1 per cent carbon dioxide bicarbonate medium to oxygen tension remains to be studied. One specific difference between the buffers was observed, namely, the ability of succinate to increase the oxygen uptake in 1 per cent carbon dioxide-bicarbonate but not in phosphate. Greig, Munro, and Elliott (1939) found that 0.01 M succinate did not increase oxygen uptake by ox retina in the presence of 5 per cent carbon dioxide and the absence of glucose. In our experiment with succinate in bicarbonate, glucose was present and only 1 per cent carbon dioxide was used.

A striking phenomenon noted in the present study was the observation that the oxygen uptake of a nearly intact tissue in the presence of glucose could be doubled by raising the carbon dioxide tension from 1 per cent to 5 per cent. The observation supports the possibility that carbon dioxide may be important at an intermediate stage in metabolism for the synthesis of dicarboxylic acids. This possibility was first suggested in connection with recent developments in the study of the Krebs cycle by Krebs and Eggleston (1940), Evans and Slotin (1940), Wood *et al* (1941), and Solomon *et al* (1941). The subject has been reviewed by van Niel *et al* (1942), and Evans (1942).

It is questionable whether the decrease in respiration of retina with 20 per cent  $CO_2$  at pH 7.48 is related to the anesthetic effect of 20 per cent  $CO_2$  in the

inspired air (Loewy, 1923, Leake and Waters, 1929, and Solomon, Kaufman, and D'Elseaux, 1931) Work in progress indicates that the metabolism of cerebral cortex and medulla oblongata is not depressed by 20 per cent  $\text{CO}_2$  at pH 7.48

Wallace and Hastings (1942) have presented data leading to the conclusion that in mammalian skeletal muscle the bicarbonate ion does not normally cross the cell wall. The question is raised whether the bicarbonate ion penetrates the cell wall of retina under the conditions of the present experiments as easily as carbon dioxide. The sensitivity of lactic acid production to pH, and the insensitivity to an increase of buffered  $\text{CO}_2$  from 5 per cent to 20 per cent have been shown in Table I. If one assumed from the sensitivity of glycolysis to external pH that glycolysis is also sensitive to changes in pH within the cell, then the fact that glycolysis was not affected by the above mentioned increase in the carbon dioxide-bicarbonate system may mean that  $\text{CO}_2$  and bicarbonate penetrate, if at all, at equal rates. If  $\text{CO}_2$  entered the cell but not bicarbonate, then the cell would turn acid and glycolysis would be inhibited

#### SUMMARY

The metabolism of rat retina was found to be sensitive to the concentration of the carbon dioxide bicarbonate buffer system. Increasing the carbon dioxide from 1 per cent to 5 per cent at constant pH nearly doubled both respiration and glycolysis. Increasing the carbon dioxide at constant pH from 5 per cent to 20 per cent had no effect on glycolysis, but depressed the  $Q_{O_2}$  from 31 to 19.

In a medium containing glucose and the 1 per cent carbon dioxide-bicarbonate buffer, the addition of succinate increased the  $Q_{O_2}$  from 12 to 26, without affecting glycolysis. In a medium containing glucose and phosphate, succinate had no significant effect.

We are indebted to Dr Fritz Lipmann for many helpful suggestions during the course of this study and to Miss Anna Murphy for her careful assistance with the chemical determinations.

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# THE INFLUENCE OF AMINO ACIDS ON THE REACTIVATION OF YEAST INVERTASE

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In a previous paper (1), the influence of some fifteen proteins on the reactivation of yeast invertase was described. Since certain proteins of a non enzymatic nature partially inhibited the reactivation, it appeared interesting to study the effect of amino acids on the reactivation.

The experimental details used in this investigation were the same as those previously described, except that the stock solution of each enzyme preparation was made up so that the phosphate-citrate buffer concentration was 0.2 M, instead of 0.015 M.

Three preparations which represent extremes in the degree of purification of invertase were used. These were (1) a commercial preparation sold under the trade name of convertit, a product of the Wallerstein Co., of New York, which contains relatively larger amounts of extraneous material than the other preparations, (2) RN<sub>a</sub>DKD, and (3) a highly purified preparation R<sub>a</sub>DKDA (S)DAD (preparations 2 and 3 having been made according to the method of Lutz and Nelson (2)). The symbols represent the following: R = brewery yeast obtained through the kindness of Jacob Ruppert Brewery, N. Y., R<sub>a</sub> = dilute acid autolysis of brewery yeast (2), RN = neutral autolysis (2), a = alcohol precipitation, D = 24 to 48 hour dialysis, K = kaolin adsorption, (S) = soluble after saturating with ammonium sulfate (2), A = alumina adsorption.

Since the quantity of preparation 3 on hand was limited, most experiments (unless otherwise indicated) were carried out at least in duplicate using preparations 1 and 2. Each result in the following section is therefore an average of 4 determinations. Before reactivation experiments can be done, it is necessary to determine the optimum pH of reactivation for each preparation, and to adjust the alkali for reactivation to obtain this pH because at this point minimum change in velocity occurs with slight change in pH.

## *Effect of Compounds not Containing SH or S S Groups*

For these experiments the initial invertase strength was diluted to give a reactivation velocity of from 0.03 /minute to 0.07°/minute. 10 mg. of the compound was used in each experiment. (The reason for using this quantity will be given in a later section.) Accelerations or inhibitions up to 10 per cent

are of no significance in this type of experiment. The results for the mono-amino acids were as follows: for *dl*-alanine, an inhibition of 5 per cent, glycine, 6 per cent acceleration, *d*-glutamic acid, 4 per cent inhibition, hydroxyvaline, 10 per cent acceleration, *l*-leucine, 2 per cent acceleration, phenylalanine 3 per cent acceleration, *dl*-methionine, 10 per cent acceleration, *l*-tryptophane, 2 per cent acceleration, *l*-tyrosine, 7 per cent inhibition, valine, 6 per cent acceleration, and *s*-benzyl-homocysteine, 14 per cent inhibition. Using the dipeptides glycyl-glycine and *dl*-alanyl-glycine, accelerations of 10 and 8 per cent respectively were obtained. For the tripeptides di-glycyl-glycine gave 8 per cent acceleration, alanyl-leucyl-glycine 0 per cent, and glycyl-*dl*-leucyl-*dl*-alanine, 2 per cent acceleration. It can, therefore, be stated that these 16 compounds did not appreciably affect the reactivation of yeast invertase.

#### *Influence of Cysteine, Homocysteine, and Reduced Glutathione*

However, the average of the reactivation experiments (using all three invertase preparations) with cysteine resulted in an acceleration of 230 per cent, while reduced glutathione (on invertase preparations 1 and 2) resulted in an acceleration of 159 per cent. When the same quantity of cysteine was left in contact with invertase at pH 4.7 and 6.0 for 75 minutes (the same length of time as in the reactivation experiments), an inhibition of 0.9 per cent was obtained. Glutathione added to invertase solution at pH 4.6 for the same length of time resulted in an inhibition of 4 per cent. Thus, cysteine and glutathione did not alter the velocity of native invertase, but resulted in greater velocities in the reactivation experiments. Attention was therefore directed towards the effect of some SH and S-S compounds on reactivation.

Other thiol compounds and their accelerative effects on the reactivation were as follows: homocysteine, 290 per cent, thioglycolic acid, 90 per cent, and thiophenol, 220 per cent. (Because of the slight solubility in water of homocysteine and thiophenol, each of these was first dissolved in 1 ml. of 95 per cent alcohol. Also, 1 ml. of 95 per cent alcohol was added to the control experiment.)

Then cystine, oxidized glutathione (3), and homocystine were added to separate portions of invertase at pH 4.7 and these solutions were allowed to stand for 100 minutes. Accelerations of 6, 0, and 0 per cent were noted, respectively. However, the inhibitions obtained with each of these compounds in the reactivation experiments were as follows: with 10 mg. cystine, 52 per cent, 5 mg. oxidized glutathione, 60 per cent inhibition, and 10 mg. homocystine, 85 per cent inhibition. Thus, an antagonistic effect on the reactivation velocity was obtained by the use of the above SH and S-S compounds. (Because of the insolubility of cystine and homocystine each was first dissolved in separate portions of the HCl used for inactivation.)

When the H of homocysteine was blocked by a methyl or a benzyl group the accelerative effect was destroyed. It will be remembered that methionine

caused a 10 per cent acceleration and that *s*-benzyl homocysteine gave an inhibition of 14 per cent

In this connection, it is interesting to mention some references to the growing literature on the effect of SH compounds on various native enzymes. Grassmann and Dyckerhoff (4) showed the activating effect of SH on yeast proteases. Shortly thereafter Waldschmidt Leitz *et al* (5, 6) reported a similar activation of mammalian tissue proteases. They found an activator which increased as autolysis proceeded, and this was later identified as glutathione (7). Anson (8) showed that polypeptidase is activated by cysteine. Fruton, Irving and Bergmann (9) concluded that splenic cathepsin is a multiple proteinase, some of whose factors require sulfhydryl activation, and some of which do not. Rapkine (10, 11) showed that the enzyme which catalyzes the oxido-reduction between triosephosphate and pyruvate is inactivated by oxidation with oxidized glutathione. If excess oxidized glutathione is dialyzed away the enzyme can be reactivated by reduced glutathione or cysteine, the latter being more rapid in its action. Bailey and coworkers (12) conclude that one of the enzymes involved in the primary cleavage of tissue proteins is activated by cysteine, thiocresol, and thioacetic acid.

The invertase experiments reported in this paper differ in that the sulfhydryl and disulfide compounds mentioned did not alter the velocity of native invertase. However, these same sulfhydryl compounds resulted in a greater velocity in the reactivation experiments, whereas the disulfide compounds inhibited the reactivation of invertase.

Bailey and coworkers (12) demonstrated that at the same pH at which cysteine increased the rate of hog liver autolysis (pH 4), the autolysis in the presence of a quantity of  $KIO_3$  sufficient to abolish the SH reaction was considerably reduced. In view of these facts, it was decided to try  $KIO_3$  on invertase. 10 mg of  $KIO_3$  did not affect native invertase but inhibited the reactivation to the extent of 88 per cent.

#### *Addition of Cysteine at Different Points in the Inactivation and Reactivation Process*

The following question then presented itself: What is the effect of adding cysteine at different points in the inactivation and reactivation process? This series of experiments was carried out using RNaDKD. When cysteine was added 1 minute after inactivation an acceleration of 230 per cent was obtained but when added 29 minutes after inactivation (and therefore 1 minute before reactivation) the acceleration was 181 per cent. The control velocity in each case was  $0.042^\circ/\text{minute}$ .

An increase in the reactivation rate is obtained whether the cysteine is added to the invertase before inactivation, immediately after inactivation, and immediately before reactivation. The longer the cysteine remains in contact with the inactivating enzyme the greater the velocity of the reactivated invertase.

This seems to suggest some definite chemical reaction between the cysteine and the product or products obtained on acid inactivation of the yeast invertase, or perhaps a reaction between cysteine and some possible inhibitor of reactivation

*The Effect of Varying Quantities of Glutathione on the Reactivation Velocity*

The results are shown in Table I. Preparation II was used in this series. The control rate was 0.06°/minute. The largest accelerative effect under the experimental conditions used is in the neighborhood of 10 mg of glutathione.

TABLE I

Glutathione	Acceleration
mg	per cent
10	141
5	126
2	93
0.5	15
0.4	2

TABLE II

Rate of reactivated enzyme	Acceleration
	per cent
0.037	267
0.042	232
0.06	152
0.106	68
0.286	39
0.310	30

For this reason it was not found necessary to use larger quantities in the reactivation experiments. Likewise, it had previously been found (1) that wherever inhibitory effects of proteins were obtained, a quantity beyond 10 mg did not appreciably alter the reactivation rates.

*The Effect of Control Rate on Per Cent Acceleration*

Finally, it was decided to study the effect of using the same quantity of accelerant (10 mg cysteine) and to reactivate solutions of varying enzyme concentration, in order to obtain different reactivation rates. The results are listed in Table II. Preparation I was used in this set of experiments. It can be seen that the lower the rate of reactivated control, the greater the acceleration.

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Van Camp Laboratories, California, for several amino acids.

#### SUMMARY

1 Some 16 amino acids (not containing SH or S-S groups) did not affect the reactivation of yeast invertase inactivated by acid.

2 Cysteine, reduced glutathione, homocysteine, thiophenol, and thioglycolic acid accelerated the reactivation of yeast invertase

3 Cystine, oxidized glutathione, and homocystine inhibited the reactivation of yeast invertase.

4 The compounds mentioned in 2 and 3 did not affect native invertase

5 The use of compounds in which the H of the SH group of homocysteine was substituted by a methyl or benzyl nullified the accelerative effect.

6 The longer the cysteine remained in contact with the inactivating enzyme, the greater was the velocity of the reactivated invertase.

7 The per cent acceleration by cysteine is inversely proportional to the control rate.

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# DIFFERENTIAL SENSITIVITY OF PROPHASE POLLEN TUBE CHROMOSOMES TO X RAYS AND ULTRAVIOLET RADIATION\*

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## PLATE 4

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### INTRODUCTION

The sensitivity of a cell to radiation depends in a large measure on the sensitivity of its nucleus (9). The sensitivity of the nucleus in turn varies with the stage of division and the rapidity of division (2, 3, 8, 12, 15, 17, 23, 25). The data of the various workers are not entirely in agreement, most stages of division, with the exception of interphase, being reported as the most sensitive or the most resistant. This disagreement stems in part from the variety of organisms used and the type of division (mitosis or meiosis) tested, and in part to two factors which can be obviated through the use of suitable techniques. These factors are the lack of a specific criterion for measuring sensitivity, and the difficulty of determining exactly the stage of division at which radiation is applied.

Two techniques, the pollen tube technique (21) in plants, and tissue culturing (4) in animals, are available which can remove most of the difficulties encountered in a problem of this sort. The former technique has been employed in this study. Because of the regularity of nuclear development in the pollen tubes, particularly in the 1 to 4 hour period after germination in *Tradescantia*, it is possible to ascertain with a good deal of accuracy the varying sensitivity of the chromosomes (as regards both primary and secondary effects) as it is correlated with changes in the developing nucleus. At the same time, the technique renders feasible a further comparison of the effects of x rays and ultraviolet radiation on mitotic chromosomes, with chromosome breakage employed as the criterion of sensitivity.

### Material and Methods

The clonal line of *Tradescantia paludosa* Anders. and Woodson used in this study was the same as that used in other x ray and ultraviolet studies (23). The slides were prepared according to the pollen tube technique (21), with enough colchicine, replacing acenaphthene dissolved in the sugar-agar-gelatin medium to give an 0.001 per cent solution. The colchicine replacement is advisable because of its ready and cer-

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tain action, and because it facilitates the chromosome analysis by spreading them widely in the pollen tube. Fixation was carried out approximately 24 hours after the pollen was sown. Since both x-rays and ultraviolet tend to impede prophase development, the greater the dose given, the longer the pollen tubes were allowed to grow before fixation. In order to keep the agar from drying out during the exposures to radiation, the slides, during exposure, were kept in a moist chamber having a water-filled cell of fused quartz as a window.

X-ray treatment was delivered by a Coolidge tube (60 kv, 10 ma), with the pollen tubes 10 inches from the center of the target. The dose was 123.6 r/minute, measured by a Victorean dosimeter.

TABLE 1  
*Effects of Ultraviolet (2537 Å) at Successive Prophase Stages (Taken in Part from Swanson (23))*

Time	Total chromosomes	Deletions	Total breaks
<i>hrs</i>			<i>per cent</i>
0	1368	20	2.52
1	1708	54	3.16
2	1728	92	5.32
3	1404	57	4.06
4	1020	26	2.55
5	1134	13	1.14
9	1236	7	0.56
11	576	2	0.35
13	1002	2	0.20
15	1470	3	0.21

For the ultraviolet treatments, two low pressure mercury arcs, both of the Hanovia SC-2537 type, were used. Both yielded approximately 85 per cent of their radiation at wave length 2537, while the greater portion of the remainder was above the genetically effective region of the spectrum. Most of the ultraviolet data (Table I) were collected while the writer was at the University of Missouri (23, Table 6), and the light source employed operated at 5000 volts. At these laboratories, the data at the 0 and 1 hour stages, as well as checks on other stages, were obtained from a similar light source but one operating at 7500 volts. Comparable doses were utilized to permit the combining of the two sets of data.

The writer wishes to express here his sincere appreciation to Dr. J. C. Clark of the Physics Department of Michigan State College for technical equipment and assistance.

#### OBSERVATIONS

##### *Differential Sensitivity to Ultraviolet*

As demonstrated in previous experiments (22, 23) ultraviolet can produce, in the generative nucleus, only chromatid deletions. It is entirely possible that these deletions, through a delayed illegitimate fusion, may result in chromatid translocations at some later stage in division than metaphase, but up

until that time, no evidence has been forthcoming which would indicate otherwise. That this is so results probably from the fact that broken ends induced by ultraviolet are more stable than those arising spontaneously or those induced by the more ionizing types of radiation (11), although ultraviolet induced translocations have been reported (19, 20)

The changing rate of deletions produced in the prophase stages of the generative nucleus indicates that secondary changes in the chromosomes, arising progressively in prophase, exert a profound influence on the rate of breakage. From the 2 hour stage on, a continuous decline obtains, the chromosomes become inherently more resistant as prophase advances. At 11 hours after the pollen grains are sown, there is no appreciable increase over the spontaneous rate. No visible deletions therefore are produced at these later stages by the incident energy. The technique unfortunately does not permit a study of the next cell division so that it cannot be stated with certainty that no breaks of any kind are produced at late prophase which might be realized at later divisions.

The 0 and 1 hour stages show a decreased rate of breakage as compared to the 2 hour stage. It would thus appear that the generative nucleus in its resting stage and earliest prophase is less sensitive to the effects of ultraviolet than are the later stages. Two factors, however, are apt to prove these data misleading. *Tradescantia* pollen has a tendency to clump at anthesis, thus rendering it difficult to dust it onto a slide in an even monolayer. There will be some shielding by the top pollen grains, thus materially reducing the amount of incident energy reaching the lowermost nuclei. Also, and this is very probably the more important factor of the two, the generative nucleus does not usually emerge from the pollen grain to pass into the tube until after the 1 hour period, necessitating thereby radiation through the wall of the grain. Uber (24) has demonstrated that while the transmission curve for pollen walls in maize has a maximum around 2500 Å, the actual transmission at this wave length is only 30 per cent. Although nothing can be said at present concerning the transmission by the pollen wall in *Tradescantia*, it is reasonable to assume that since it is heavily pigmented it would considerably reduce the amount of energy reaching the generative nucleus. It seems likely therefore that the data do not give a true picture of the sensitivity at the 0 and 1 hour stages, and it is suspected though not proved, that the resting stage is more sensitive, so far as chromosome breakage is concerned than are the prophase stages.

When the nucleus has passed into the tube transmission would be at its highest value for the nucleus is now free of the pollen wall, and the pollen contents are considerably diluted by the uptake of water. It should be pointed out, however that Uber (24) has shown the pollen contents exclusive of the wall, to have again a maximum at about 2500 Å, but with an actual transmission of only 20 per cent.

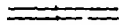
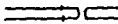
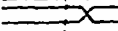
*Differential Sensitivity to X-Rays*

In comparing the effects of x-rays and ultraviolet on the pollen tube chromosomes (23), it was pointed out that during the 2 to 4 hour period after germination the effects of these two types of radiation were exactly opposite. The sensitivity to ultraviolet decreased while to x-rays it increased. This analysis has now been carried to a point where a comparison can be made for the whole prophase stage (Tables II and III), and it can now be stated that the differential reaction found at the 2 to 4 period is absent at later stages, both ultraviolet and x-rays showing a continued drop in breakage rate as prophase approaches metaphase.

TABLE II

*Effects of X-rays at Successive Prophase Stages Exposure 3 Min Dose 370 r*

Time	Total chromosomes	Single* deletions	Double* deletions	Translocations*	Total breaks
hrs					per cent
0	2400	8	20	12	2 16
1	1596	68	46	18	9 46
2	2532	119	49	47	10 35
3	2430	123	30	57	10 98
4	2136	135	28	54	12 72
5	1800	102	18	18	8 66
6	1872	56	0	24	5 55
7	1290	10	5	0	1 16
8	1872	6	6	0	0 62
10	1224	3	0	0	0 24
15	786	1	0	1	0 38
Control	2520	3	1	1	0 23

\* Single deletion Double deletion Translocation 

Taking the per cent of total breaks as an indication of the degree of sensitivity (Table II), the 4 hour period represents the point of maximum sensitivity. A gradual decline occurs at later stages until at the 10 hour period the breakage rate does not exceed that produced spontaneously. The later stages of prophase thus respond identically to both types of radiation.

Table III presents the various types of x-ray breaks in terms of per cent. Single deletions and translocations follow a similar trend, with their maxima at the 4 hour period. The close relationship of single deletions to translocations suggests that the former contribute to the production of the latter, thus lending additional support to the breakage-first hypothesis of Stadler. The double deletions reveal a rise at the 1 hour period, but the decline begins imme-

diately after Sax (13) has shown that similar trends are to be found among x ray aberrations in *Tradescantia* microspores. As Sax also emphasizes, the changes, with the exception of the sharp mutual rise following germination, are gradual in nature and correspond to the gradual changes taking place in prophase

TABLE III

*Effects (in Per Cent) of X Rays at Successive Prophase Stages Exposure. 3 Min.  
Dose 370 r*

Time	Total chromosomes	Single deletions	Double deletions	Translocations	Total breaks
hrs		per cent	per cent	per cent	per cent
0	2400	0.333	.83	1.00	2.16
1	1596	4.26	2.88	2.25	9.46
2	2532	4.71	1.93	3.71	10.35
3	2430	5.06	1.23	4.69	10.98
4	2136	6.32	1.35	5.05	12.72
5	1800	5.66	1.00	2.00	8.66
6	1872	3.00	0.00	2.55	5.55
7	1290	0.77	0.38	0.00	1.16
8	1872	0.32	0.32	0.00	0.62
10	1224	0.24	0.00	0.00	0.24
15	786	0.13	0.00	0.25	0.38

#### *The Correlation of Sensitivity with Prophase Behavior*

In attempting the interpretation of any biological mechanism, it is desirable, if not essential, to seek relationships between form or structure on one hand and behavior on the other. When a relationship exists, and both are variable, then their variabilities should likewise show correlation. This is true for chromosome breakage, for, as has been pointed out (13, 15, and others), the mechanism of breakage through radiation is conditioned to a considerable extent by the structure and behavior of the chromosomes at the time of radiation or shortly thereafter. This can be clearly demonstrated in the generative nucleus because of its readily traceable progressive development, particularly in the early prophase stages where changes in sensitivity vary greatly. In later stages, however, there is an overlapping of stages which makes too close a comparison inaccurate and undesirable.

The generative nucleus in the pollen grain before germination (0 hour period) is elongated, highly chromatic, and compact (Fig 1). There can be but a minimum of movement in such a condensed nucleus, and conditions are such that external stresses are not operative, thus favoring the *status quo* of the chromosomes. On the "direct hit" theory of Sax (14) this would signify a high rate of reunion. If x rays produce potential breaks (10), then the realization of

breaks would be low. In either event, a low breakage rate obtains due to the absence of secondary factors. With the beginning of germination, the nucleus elongates and eventually passes into the pollen tube. Its length has by this time increased considerably (Fig 2), and although there is some decrease in the width, the length increase is most likely made possible by an uptake of water, a phenomenon long known to accompany nuclear division. The initial 4-fold increase in x-ray breaks at the 1 hour period (Table II) is undoubtedly traceable to the stresses involved in the uptake of water, with the subsequent opening up of the relic coils as well as in the elongation of the nucleus and its movement as a whole in the tube. Although the individual chromosomes are distinct enough at the 1 hour stage there is no indication of the presence of a new somatic coil. This coil, however, is well established by the 4 hour period (Fig 3), and other observations show that spiralization begins at about the 2 hour stage. Since the chromatid, and not the chromosome, is the unit of coiling, and is independent, to a certain degree, of its sister chromatid, it seems reasonable to assume on *a priori* grounds that a reduction would occur in number of double deletions while an increase would be found in the single deletions. Since the single deletions probably contribute to the translocations, a rise would be expected in the latter. This is what is found. The period of greatest activity (loss of relational and relic coils, initiation of somatic coils) coincides with the period of greatest sensitivity. Coiling during mid and late prophase (Figs 4 to 6) is a more leisurely affair, and movement is restricted. Sax (13) has stated that double deletions can be produced by x-ray hits in chromosomes that are visibly split. In the generative nucleus, the split is first evident at the 2 hour stage, and although at this stage the per cent of double deletions is low when compared to other types of breaks, they are nevertheless produced in appreciable numbers, thus bearing out Sax's contention that a single hit can traverse the distance involved in breaking a double-stranded thread.

At the later stages of prophase, other factors must exert an influence on chromosome breakage, otherwise it is difficult to explain the absence of even single deletions. What these factors are can only be conjectured at this time, but undoubtedly the development of the matrix and the nucleic acid accumulation (5, 6) both act as stabilizing influences.

#### DISCUSSION

The data presented reveal that while a difference exists in the reactivity of the chromosomes to ultraviolet and x-rays during early prophase, the later stages (most of the middle and all of the late prophase) show a gradually decreased sensitivity to both types of radiation. This, in part, is in reasonable agreement with the data of Sax and Swanson (15) on *Tradescantia* microspores. It is not, however, in agreement with the more recent data of Sax (13), also on microspores, in that a fall rather than a continued rise of single deletions and

translocations is found from mid prophase on. The disagreement can result from two factors (1) a differential behavior of the microspore and pollen tube chromosomes to x rays, or (2) a difference in the interpretation of prophase stages. The second factor is probably the more important of the two. Sax considers prophase to begin with the effective splitting of the chromosomes, a condition which may or may not be true since in the microspores there is no method of determining exactly the stage of division irradiated. Again, if, in the generative nucleus, this criterion were used then prophase would begin 2 days before anthesis, since at this time chromatid breaks can be obtained. On the other hand, prophase in the pollen grain and tube appears no different than in other mitotic cells with the one exception that the generative nucleus is compact and elongated rather than large and spherical.

The impression is gained, from development studies of the generative nucleus, that two factors determine the change in sensitivity to x rays in the 1 to 4 hour period. These are the uptake of water which leads to an elongation of the nucleus, and hence leads to much chromosome movement, and the initiation and continuation of the spiralization cycle. Later stages find the spiralization tempo much reduced, and the chromatids widely separated, resulting in fewer breaks. It seems unlikely, however, that the inability of x-rays, at the dosage used, to produce breaks of any detectable kind in late prophase can be adequately explained by the above factors unless it is assumed that breaks are produced but are not realized until the next cell division. Unfortunately this technique does not permit such an analysis.

That the activity of the chromosomes in prophase is inadequate to explain the decreasing sensitivity to ultraviolet has been emphasized (23). Also McClintock (11) has suggested, from genetical data, that the ultraviolet breaks are relatively stable, indicating that not only are the breaks not visibly realized but that in all likelihood they are not being produced at all. To state it otherwise, the diminishing rate is not due to an increasing rate of reunion. It becomes increasingly evident therefore that changes inherent in the structure of the chromosome condition its sensitivity to ultraviolet.

Since the efficacy of ultraviolet results from a selective absorption dependent upon the local properties of the irradiated substances, it is essential that the nature of these inherent changes be known if the mechanism of chromosome breakage is to be understood. While the chemical nature of the chromosome, and the alterations in its molecular structure which it undergoes while in division, are as yet but partially comprehended, there are three tentative aspects of the problem which, in relation to chromosome breakage, bear further investigation. These are (1) the changes in nucleic acid attachment, (2) the behavior and development of the matrix, and (3) further splitting of the chromosome. Their relative importance at this time is conjectural.

The division cycle of a nucleus is accompanied by a cycle of attachment

and detachment of nucleic acids to and from the protein framework of the chromosome (5, 6) Ultraviolet absorption techniques as well as staining methods show the maximum attachment to occur at metaphase, and the maximum detachment at interphase Signer, Caspersson, and Hammersten (18) have further demonstrated that the molecules of nucleic acid polymerize in long chains which parallel the longitudinal axes of the backbone proteins with a spacing nearly identical with that of the polypeptides comprising the proteins (1) This suggests that the nucleic acids, as they accumulate, act as a protecting sheath to the encased proteins, thus preventing their dissociation by the action of ultraviolet Since the accumulation continues up to metaphase, so the resistance will similarly increase This may serve to explain the approximation of Stadler's (19) ultraviolet deficiency data in maize to a nucleic acid absorption curve Additional support has been furnished by Greenstein, Jenrette, and Hollaender (7), who show that the action of ultraviolet on sodium thymonucleate is one of depolymerization It is thus possible that depolymerization of the nucleic acids attached to the proteins of the chromosome is a necessary step before breakage can occur Whether an explanation of the failure of x-rays to produce fewer and fewer breaks in late prophase (Tables II and III) is to be sought in a mechanism of this sort cannot at present be stated, although in view of Bozeman's (2) recent data, this seems unlikely

The rôle of the matrix in chromosome breakage is problematical in view of our limited knowledge of the physical features and function of this structure Metz and Bozeman (12) consider x-rays to affect the viscosity of the matrix There is some evidence (23) that the matrix increases in dimension as the division cycle approaches metaphase, acting thus as a stabilizing factor in maintaining the integrity of the chromosome Schultz (16) has demonstrated the presence, in *Drosophila* mitotic chromosomes, of a matrix of a complex protein bound to the chromosome proper by ribose nucleic acid linkages Although the pattern of chromosome-matrix relationship remains obscure for the time being, the mere presence of this structure and the fact that a chromosome break must necessarily involve the matrix as well as the chromosome proper, emphasizes that it must be taken cognizance of in any consideration of the mechanism of x-ray or ultraviolet breakage

A third factor which may exert an influence on the rate of ultraviolet breaks is the number of subdivisions in the chromosome at the time of irradiation Sufficient evidence has accumulated from many varied sources to state safely that the interphase chromosome is double regardless of its reaction to x-rays This doubleness can be best seen in anaphase, so division into half-chromatids must have taken place at metaphase or earlier That half-chromatids are present in prophase is indicated by this study In the first place, numerous breaks are found which extend only a part way across the diameter of the chromatid, suggesting that only one of the two half-chromatids had suffered

breakage. Also, one unequivocal and several less certain instances of half chromatid translocations have been detected at the 2 hour stage following x radiation. In the single clear case, the half-chromatids at the point of breakage could be traced with ease and clarity. If these observations are correct, and it is difficult to conclude otherwise, then two points become clear first, that since ultraviolet is a dissociation agent, with a limited sphere of action, it is to be expected that most of the breaks produced would involve half-chromatids, and would therefore not be detected until the following cell division, and second, that ultraviolet can break a double-stranded thread, implying that other factors than the mere "hit," or absorption, are necessary for the realization of a break in the chromosome. This latter implication is supported by Stadler's (19) data on endosperm deficiencies when he shows that although the majority of ultraviolet deficiencies are fractional, some of them are entire, involving in this manner both strands of a split chromosome.

From the above discussion it is apparent that the explanations presented in interpreting the ultraviolet data are to be regarded as suggestive only, and must await further study. Their validity will be tested as our knowledge of the chemistry of the chromosome and the chemistry of radiation increases.

#### SUMMARY

1 Through use of the pollen tube technique it has been possible to study the sensitivity of prophase stages to x rays and ultraviolet and to correlate the varying sensitivity with changes in the generative nucleus of *Tradescantia*.

2. Sensitivity to ultraviolet decreases from the 2 hour stage until at 11 hours after germination there is no further production of breaks. The 0 and 1 hour stages show a decreased sensitivity over the 2 hour stage but it has been suggested that this is not due to a decreased sensitivity but to shielding by the pollen wall.

3 Sensitivity to x rays rises to a peak at the 4 hour stage, but then subsides until no breaks are realized (at a dose of 370.8 r) after the 10 hour stage. In this respect the effects of x rays and ultraviolet are similar. Each type of x ray break shows its own individual trend.

4 Correlation of x ray breaks with changes in the generative nucleus indicates that the important events determining the sensitivity of the chromosomes to breakage are the uptake of water at the time of germination and the movement involved in spiralization. The total absence of breaks after the 11 hour stage is not understood.

5 The changing sensitivity to ultraviolet may depend on any one or all of three factors (a) the nucleic acid cycle, (b) changes in the matrix, and (c) the number of subdivisions in the chromosome. These are discussed although their relative importance is not known.



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## EXPLANATION OF PLATE 4

Figs 1 to 6 Successive prophase stages in the generative nucleus

Fig 1 Mature pollen grain prior to germination, generative nucleus above, tube nucleus below

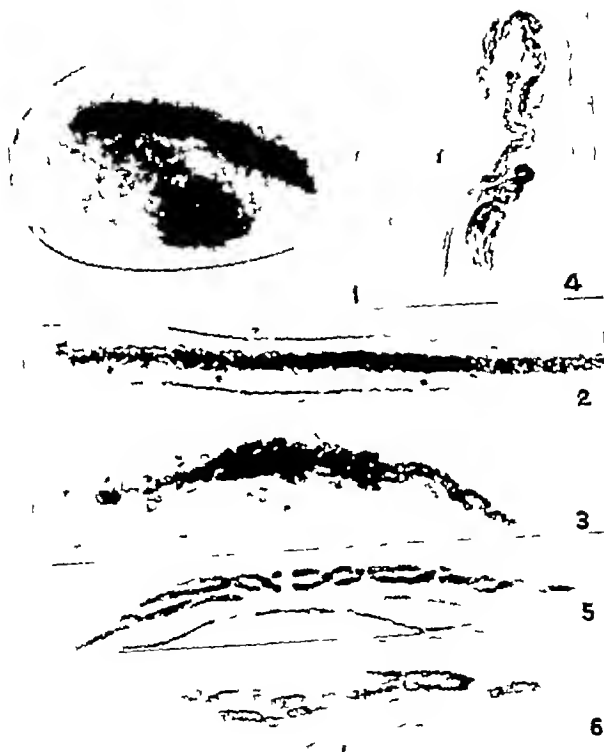
Fig 2 1 hour stage of generative nucleus

Fig 3 4 hour stage

Fig 4 6 hour stage

Fig 5 8 hour stage

Fig 6 15 hour stage



(Swanson. Sensitivity of prophase pollen tube chromosomes)

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## EXPLANATION OF PLATE 4

Figs 1 to 6 Successive prophase stages in the generative nucleus

Fig 1 Mature pollen grain prior to germination, generative nucleus above, tube nucleus below

Fig 2 1 hour stage of generative nucleus

Fig 3 4 hour stage

Fig 4 6 hour stage

Fig 5 8 hour stage

Fig 6 15 hour stage

# PHYSICAL PROPERTIES OF THE ALLANTOIC AND AMNIOTIC FLUIDS OF THE CHICK

## I. SPECIFIC CONDUCTANCE

BY PAUL ANDREW WALKER

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*(Received for publication, April 20, 1943)*

The importance of the composition of the surrounding medium to the development of the organism is well known and may be studied to great advantage in the hen's egg in which the embryo secretes its own external environment, the amniotic fluid. In the later developmental stages, when the allantois has come to surround the other components of the egg, the contents of this membrane are also of significance. The extra-embryonic fluids of the developing chick have received considerable attention in the past, but until recently the investigations have not been sufficiently systematic to afford a complete picture of the changes in the nature of these fluids or of the effects which this changing environment might exert upon the developing embryo. Most of these analytical studies have been limited to investigations of changes in the chemical composition of the fluids at different stages of development. It is equally important to secure information as to the changes in their physical properties. The present series of electrical conductance measurements under takes to supply some of this information.

Relatively few investigators have concerned themselves with the study of changes in the electrical conductance of the extra-embryonic fluids. Of these, Romanoff and Grover (1936) are the only ones who have examined this question systematically in the chick. The present work, which was completed before the appearance of their paper, has yielded slightly different results and hence is reported here.

### *Material and Methods*

The eggs used in these experiments came from a large flock of Barred Plymouth Rock pullets. Incubation was carried out at 38.5°C. in a regulation Thelco incubator. Samples of the fluids were obtained as follows. The shell and outer shell membrane were removed from the region of the air chamber and the opaque inner shell membrane rendered transparent by painting with mineral oil. It was thus possible to insert a fine glass pipette into the allantoic cavity without rupturing any of the allantoic blood vessels and withdraw a sample of the contained fluid. This sample was then transferred to a small glass vial, stoppered tightly and the vial and its contents placed in a wire rack within the temperature bath. In the case of embryos of less than 8 days' incubation, the pipette could not be inserted directly into the allantoic



Since considerable variation was apparent between chicks of the same incubation age, the idea of plotting conductance against incubation time was abandoned and instead the natural logarithms of the various wet weights (in grams) were used as abscissae. This function of the weight was used merely for convenience, the data being thus spread out in better fashion. On the same graph, however, are to be found vertical lines, more or less arbitrarily drawn, classifying the embryos secondarily according to days' incubation.

The apparatus used in the determination of the electrical conductance was the one described by Jones and Josephs (1928). Since most standard cells for conductance work are designed for relatively large volumes of fluid it was necessary to design a microcell which could be used with the small volumes of fluid found during certain developmental stages. This cell has been previously described (Walker 1938 a)

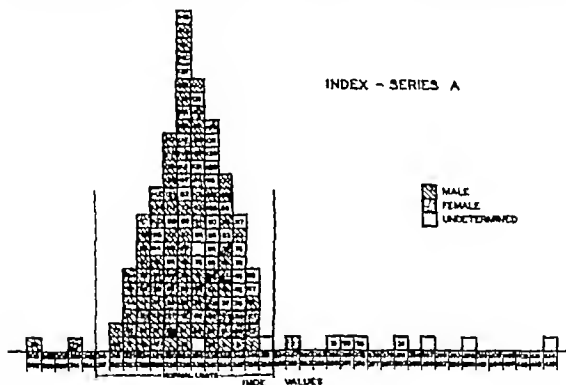


FIG 1 Histogram resulting from application of index to experimental material. Numbers refer to individual animals used.

Experiment had shown that an interval of about 20 minutes should be allowed before each measurement for the liquid within the cell to come to equilibrium with the bath temperature (20°C.) Readings were then taken at 5 minute intervals until three consecutive readings checked within 1 ohm. This represented an accuracy of at least one part in a thousand generally in fact an even higher degree. The specific conductance was then computed from the cell constant in the usual manner.

Throughout this series of experiments, the resistances were measured at 20°C a temperature chosen arbitrarily. It seemed possible that the change produced by cooling the fluids from the incubator temperature to 20° might vary in magnitude with the age of the embryo. Accordingly the fluids from two chicks, one aged 9 days and the other 16 were measured at both temperatures and the effects of this

cavity, since this membrane had not yet become fused with the chorion in the region of the air space. In such instances, more shell had first to be removed to expose the sac.

After the allantoic fluid had been sampled, enough shell was removed so that the contents of the egg could be gently poured out into a finger-bowl. A clean pipette

TABLE I  
*The Effect on Measured Resistance of Decreasing the Temperature*

Material	Resistance in ohms			Increase $\Delta R/R_{20}$
	$R_{20}$	$R_{25}$	$\Delta R$	
A37 AL F	1142	793.9	349.1	per cent 43.97
AM F	1031.5	719.7	311.8	43.32
A38 AL F	1792	1251.5	540.5	43.19
AM F	1853.5	1291.7	561.8	43.49
Average per cent increase				43.47

TABLE II  
*Average Measurements of All Embryos Used*

Age	Crown rump length	Weight	ln W	Specific conductance	
				AL.F	AM.F
days	cm	gm		mhos per cm	mhos per cm
7	2.6	0.71	1.66	0.01157	0.01328
8	3.0	1.02	0.02	0.00994	0.01319
9	3.3	1.41	0.34	0.01026	0.01316
10	3.6	1.95	0.67	0.01021	0.01323
11	4.2	2.76	1.01	0.01145	0.01357
12	4.7	3.87	1.35	0.01042	0.01348
13	5.2	5.10	1.63	0.01088	0.01334
14	5.8	7.21	1.98	0.01066	0.01297
15	6.4	10.26	2.33	0.01031	0.00811
16	7.1	13.13	2.57	0.01047	0.00826
17	7.4	15.00	2.71	0.00865	0.00883
18	7.7	17.27	2.85	0.00904	0.01014
19	8.3	21.00	3.04	0.00762	0.01221

was used in drawing off samples of the amniotic fluid, otherwise the technique was the same as described above. Precautions were taken that all glassware used in handling these samples was thoroughly clean and dry.

In order to obtain enough material in each case to fill the conductance cell (about 0.1 cc.), the range of development studied was limited. No samples were taken from embryos younger than about 7 days or older than about 19.

Since considerable variation was apparent between chicks of the same incubation age, the idea of plotting conductance against incubation time was abandoned and instead the natural logarithms of the various wet weights (in grams) were used as abscissae. This function of the weight was used merely for convenience, the data being thus spread out in better fashion. On the same graph, however, are to be found vertical lines, more or less arbitrarily drawn classifying the embryos secondarily according to days incubation.

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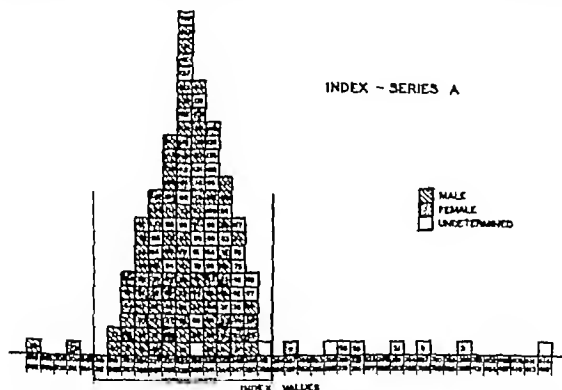


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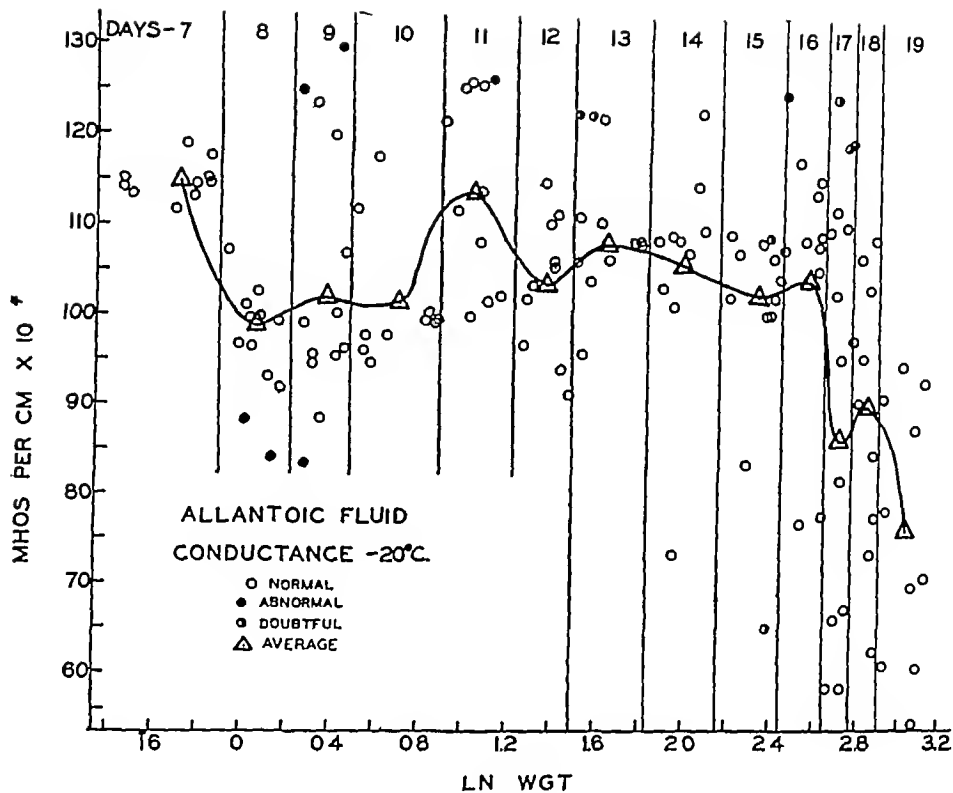


FIG 2 The specific conductance of the allantoic fluid—approximate incubation age indicated on top scale

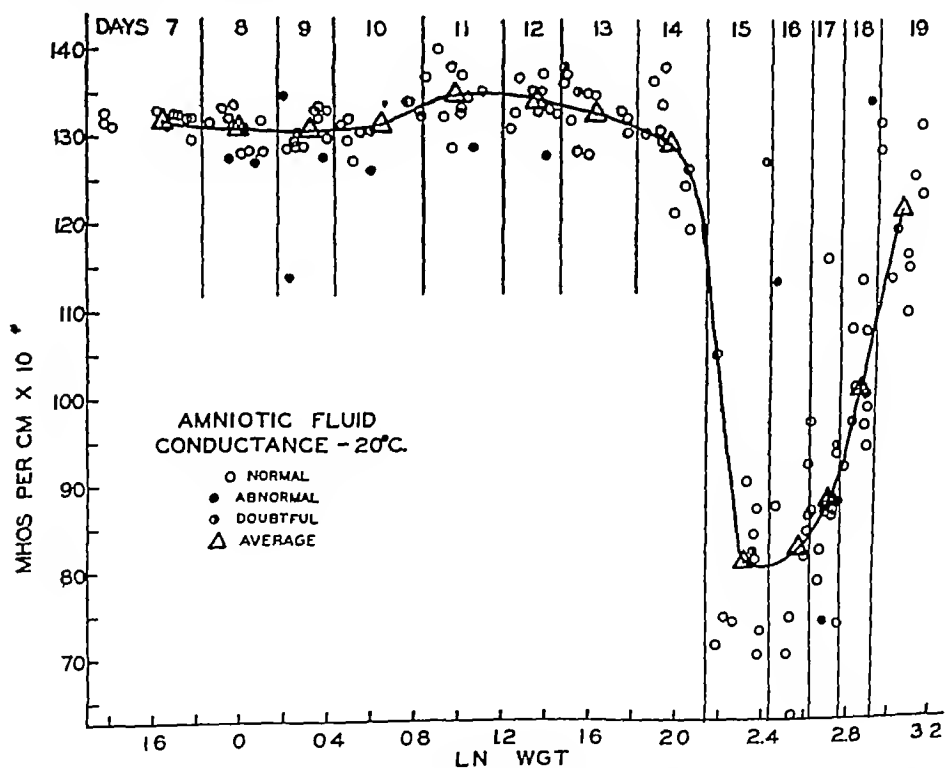


FIG 3 The specific conductance of the amniotic fluid—approximate incubation age indicated on top scale

decrease in temperature compared. The results of this experiment are shown in Table I.

The slight variation in the above percentages is insignificant in view of the known variation existing between chicks of the same age. Furthermore, since the ages of these two embryos represent rather widely separated stages it seems logical to suppose that this increase in resistance is fairly constant throughout the range of development studied. This means of course that the shape of the resulting curves has been unaltered, but merely their positions changed by this drop in temperature.

### *Experimental Data*

Specific conductances were determined for each of the two fluids upon ten chicks of each "age" within the range of development studied. Determinations upon chicks whose index value (see Walker, 1938b) lay outside the range of normality were ruled out, as were all cases in which either fluid had been accidentally lost or was thought to be contaminated in any way. Fig. 1 shows the histogram obtained after having calculated the indices for the individual chicks used in this series of experiments. The individual conductance values obtained in the case of the allantoic fluid are plotted in Fig. 2, those of the amniotic fluid in Fig. 3. In both cases the average values for each "day" are also shown. These averages are contained in Table II below. Points marked "abnormal" represent determinations on chicks whose index lay outside the range of functional normality (315-353).

### DISCUSSION

From an examination of Fig. 2, it is evident that by the 8th day of incubation, a decrease in the specific conductance of the allantoic fluid has occurred. Unfortunately, the true magnitude of this decrease is not determinable, since no measurements were made upon fluids from embryos younger than about 7 days. This decrease agrees with the observations of many workers, among them Needham (1925, 1926a, 1926b), that there occurs in the chick a succession of end-products in nitrogen metabolism: ammonia  $\longrightarrow$  urea  $\longrightarrow$  uric acid. The period of maximum ammonia production occurs quite early in development (about the 4th day), but the peak in the production of urea is not reached until the 9th day. Accordingly, it is not surprising to find a relatively high specific conductance indicating a relatively high percentage of these two dissociable compounds present at this time. Between the 7th and 8th days, the concentration of the relatively insoluble uric acid is increasing rapidly, while that of ammonia is decreasing. This is evidenced by the falling conductance values of this period. Little information is available on the important question of the amount of inorganic material present at this stage. Kamer's (1928) data show a high concentration of these substances on the 9th day, but do not tell whether or not this is the case before this time. The

decrease in conductance prior to the 9th day might be thought of as resulting from a decrease in the concentration of these readily ionizable substances

From the 9th to the 13th days, the conductance of the allantoic fluid shows rather irregular fluctuations, tending to rise slightly. There do not seem to be very significant changes here, although a slight increase in the relative ionic concentration is indicated. However, from the work of many investigators—Fiske and Boyden (1926), Targonski (1927), Fridericia (1912), Needham (1926*a*, 1931), Tomita and Takahashi (1929), Kamei (1928), Yamada (1933), and Needham, Brachet, and Brown (1935)—it is known that the total contained nitrogen (presumably in the form of uric acid) is increasing throughout this period of development. Since conductance values remain relatively stable, this must mean a corresponding increase in dissociable constituents. That these dissociable constituents are not inorganic salts is perhaps indicated by the data of Kamei (1928), however, his examination was not complete, and the results do not agree with those of Iseki (1930) on the chemical constituents at this stage. Hence, too much weight cannot be placed on these findings.

Between the 13th and 16th days the conductance of the allantoic fluid is beginning to diminish somewhat. During this time, the change-over from mesonephric to metanephric excretion is occurring in the embryo, and the changes in conductance may be linked with this phenomenon. Certainly the relative concentration of the non-dissociable uric acid is still increasing at this stage. Furthermore, the absorption of water from the contents of the allantoic sac is now beginning, although it is not nearly as marked as in the last few days of incubation.

The final descent of the conductance curve at the end of the period of development investigated is doubtless explainable on the basis of water absorption. It is known that a marked decrease in the volume of the allantoic liquid occurs at this time, and with this absorption of water would go a decided increase in the relative concentrations of all solutes and substances suspended in the fluid itself. Since uric acid and certain insoluble urates are still the chief constituents here, it is to be expected that the specific conductance of the fluid would show a pronounced decrease.

In the case of the amniotic fluid (Fig. 3), the conductance values recorded over the first period of development studied are decidedly higher than those of the allantoic liquid during this same time. This is to be expected from a consideration of investigations on the chemical constitution of this fluid. Fiske and Boyden (1926), Targonski (1927), Kamei (1928), and others have definitely shown that the amniotic fluid contains decidedly less nitrogen than the allantoic, which is, of course, receiving the end-products of the embryo's nitrogen metabolism. Kamei (1928) has also demonstrated a higher concentration of inorganic substances in this fluid. That so little change is evidenced up to the 13th day of incubation indicates that a fairly constant relationship is being maintained between ionized and un-ionized material. The slight maximum

seen at the 11th day is probably a true one, for it is shortly after this stage, according to Hirota (1894), that the perforation of the sero-amniotic connective occurs, with the attendant passage of the contents of the albumen sac into the cavity of the amnion. These observations are borne out by the subsequent precipitous fall in conductance values recorded during the 14th and 15th days. Apparently, the influx of protein material from the albumen sac takes place gradually at first, then with increasing velocity. Figures on the chemical composition of this fluid at this period (Kamei, 1928) show a maximum in total nitrogen at about this time.

After the 15th day, a rise in conductance occurs. This is coincident with the disappearance of this protein material. Schenck (1932) has pointed out that these proteins which appear in the amniotic fluid (and which can be shown to be identical in nature with those formerly present in the albumen sac) are completely ingested by the embryo, vanishing from the amniotic fluid by the 18th or 19th day of incubation. Kamei's data also show a decrease in total nitrogen during this period.

#### SUMMARY

- 1 The specific conductance of the allantoic and amniotic fluids of the developing chick has been determined over the period of incubation between the 7th and 19th days.
- 2 Changes in this property have been related to changes in the chemical composition of these two fluids.
- 3 These conductance values are of importance in that they show the relation between ionized and un ionized materials present in the two fluids.

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of these 'age' groups were tested. The index of functional normality described by Walker (1938) was again employed and Fig. 1 is the histogram obtained for this

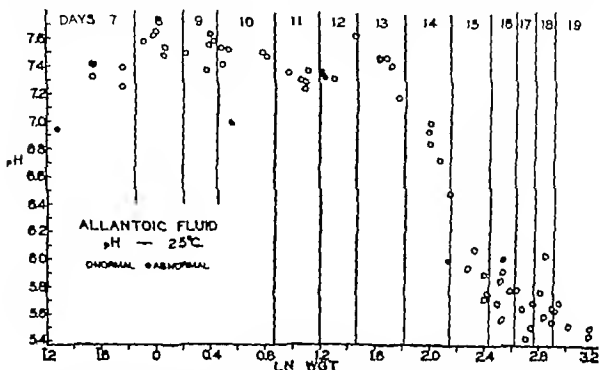


FIG. 2. pH of the allantoic fluid—measurements upon individual chicks (approximate incubation age indicated on top scale)

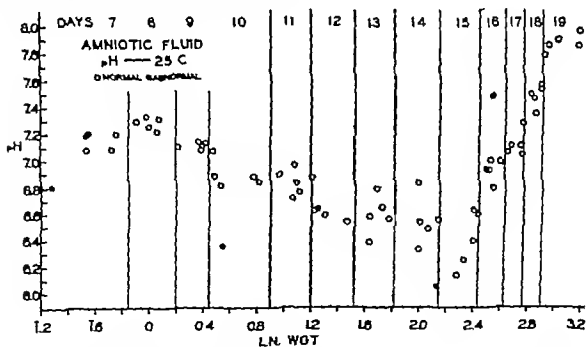


FIG. 3. pH of the amniotic fluid—measurements upon individual chicks (approximate incubation age indicated on top scale)

series of embryos. As before, the median class is that represented by embryos with index values of 333 to 335 with limits of functional normality from 315 to 353



The eggs used all came from the same flock of Barred Plymouth Rock hens. Samples of the two fluids were taken as previously described (Walker (1943)), and these were stored in stoppered glass vials within the incubator (at 25°C) for at least

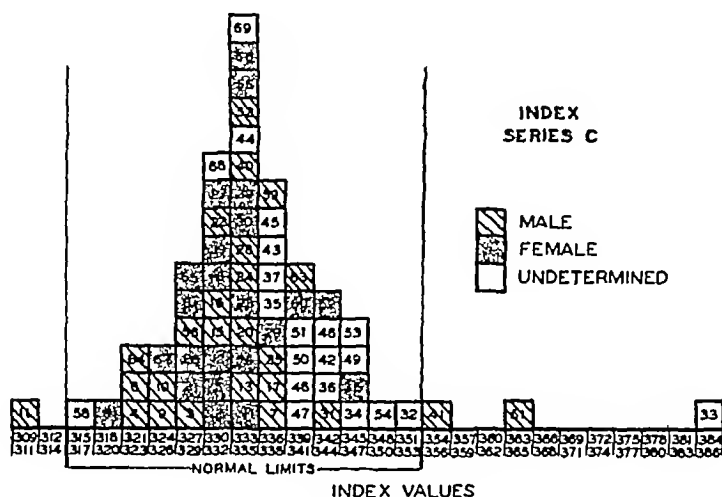


FIG 1 Histogram resulting from application of index to experimental material

TABLE I  
*Average Measurements of All Embryos Used*

Age	Crown rump length	Weight	ln W	pH	
				AL.F	AM.F
<i>days</i>	<i>cm</i>	<i>gm</i>			
7	2 6	0 68	1 63	7 36	7 15
8	2 95	1 00	0 00	7 57	7 28
9	3 3	1 44	0 36	7 53	7 11
10	3 6	1 88	0 62	7 49	6 90
11	4 2	2 89	1 06	7 31	6 83
12	4 7	3 65	1 29	7 39	6 65
13	5 25	5 41	1 69	7 39	6 58
14	5 9	7 72	2 04	6 79	6 54
15	6 7	10 69	2 37	5 87	6 40
16	7 1	12 67	2 54	5 75	6 92
17	7 4	15 22	2 72	5 60	7 12
18	7 85	17 58	2 86	5 71	7 48
19	8 4	21 13	3 05	5 55	7 87

20 minutes before use. As before, a great deal of variation was found in embryos of the same incubation age, and accordingly the material was classed primarily by wet weight. Chicks of a certain weight class have been grouped together more or less arbitrarily, to approximate the familiar "days incubation". Five individuals of each

of these "age" groups were tested. The index of functional normality described by Walker (1938) was again employed and Fig 1 is the histogram obtained for this

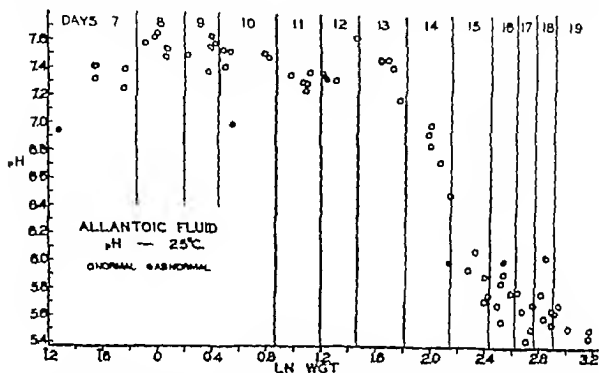


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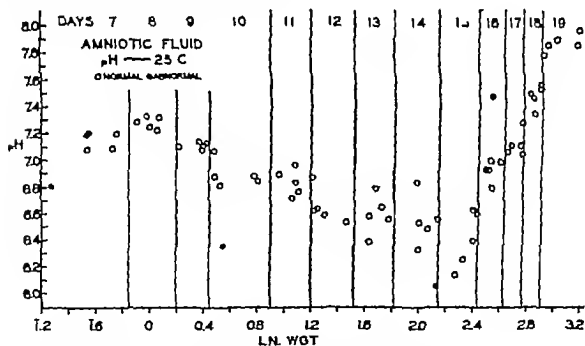


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In Figs 2 and 3 will be found the results of the pH determinations on the individual chicks used in these experiments. Average pH values for this series are shown in Table I and in Fig 4. In this last figure, the data of Aggazzotti (1913), of Buytendijk and Woerdeman (1927), and of Yamada (1933) have been included for purposes of comparison.

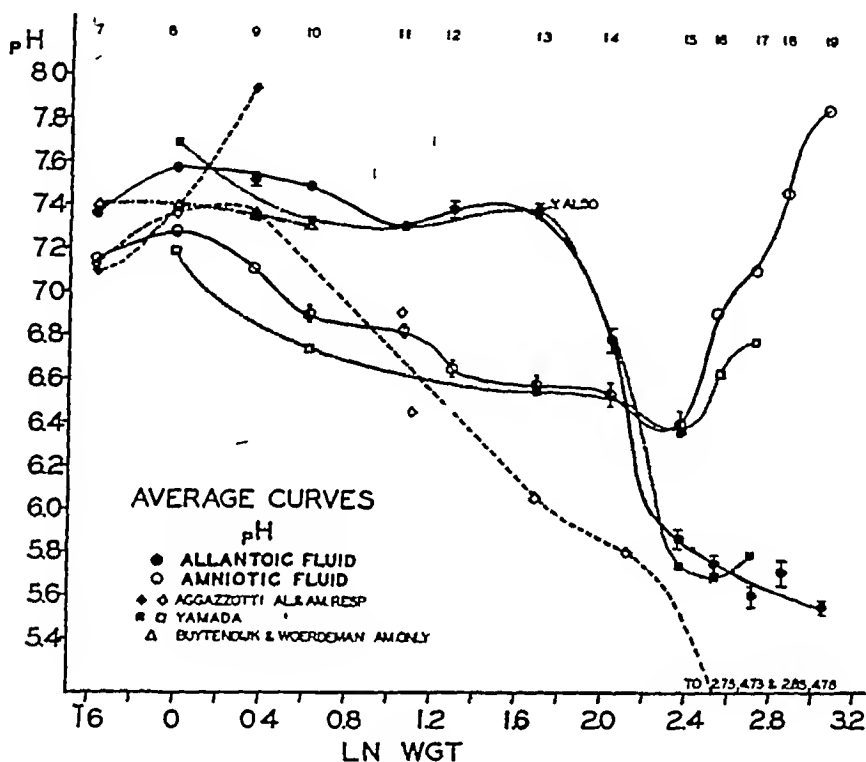


FIG 4 Average curves, hydrogen ion concentration of the allantoic and amniotic fluids—each point on the above curves represents the average values of both weight and pH of the five individuals of each "age" studied

#### DISCUSSION

In the case of the allantoic fluid, we find the hydrogen ion concentration maintained at an approximately constant level during the first half of the period of development under investigation, with pH's slightly on the alkaline side of neutrality (range 7.31 to 7.57). The hydrogen ion concentration of this fluid should in large measure be determined at these early stages by the character of the renal secretions which are being discharged into it. In this connection, Needham (1925, 1926a, 1926b) has shown that there is a succession of end-products of nitrogen metabolism in the chick: first, ammonia is produced, reaching a maximum concentration on the 4th day; secondly, urea, with a maximum on about the 9th day; and finally, uric acid. Needham

(1931a) has shown that uric acid is first excreted in the form of soluble urates, which on dissociation yield free basic ions, and these ions, together with the small amount of ammonia which is presumably still present and other unidentified basic ions, evidently counterbalance the urea<sup>1</sup> to such an extent that an equilibrium is reached which is slightly on the basic side of neutrality.

Another factor which must receive consideration here is the probable presence of carbon dioxide in this fluid (and in the amniotic fluid also). Unfortunately, the carbon dioxide content of these extra-embryonic fluids has not yet been determined, but in view of the respiratory function of the allantois, the allantoic liquid must contain a significant amount of this substance. From the results of chemical analyses, it is impossible to estimate the buffering capacities of this fluid, although the possible existence of a phosphate system is indicated. However, unless the buffering powers were rather high, carbon dioxide present in this fluid by diffusion from the allantoic blood vessels would exert a considerable effect upon its pH. This effect is not seen during the early portion of the period under investigation even though Pott and Preyer (1882) have shown that the amount of carbon dioxide produced by the embryo increases markedly with development (approximately tenfold in the period between the 7th and 19th days). These early observations of Pott and Preyer have since received abundant confirmation, as for example in the work of Murray (1927).

During the last week of incubation, the picture changes. A definite shift to the acid side becomes evident with an abrupt fall in pH from 7.39 on the 13th day to 5.87 on the 15th day, this is followed by a more gradual decrease to 5.55 on the 19th day. This marked increase in acidity foreshadows the conditions seen in adult avian urine (pH 5.0, Takamatsu (1935)), and is presumably due to a combination of the following factors. In the first place, since carbon dioxide production is continually increasing with development, there may be such an increase in the concentration of carbonic acid present in the fluid that its buffering capacity is overbalanced. Secondly, there occurs at this time a reduction in the basic constituents of the allantoic fluid because the excretory products are now being formed as free uric acid rather than its salts. Thirdly, as Fell and Robison (1934) have shown, calcification of avian cartilage and osteoid tissue begins at about the 15th day of incubation, a phenomenon which would result in a mobilization and withdrawal of calcium and phosphorus from other regions of the egg. That this would affect the extra-embryonic fluids is clearly suggested by the analyses of Iseki (1930). This loss of calcium and phosphorus from the allantoic fluid would doubtless greatly reduce its buffering powers.

Furthermore, according to Sula (1935), uric acid is less soluble in acid media.

<sup>1</sup> Urea dissociates with the formation of free hydrogen ions, according to Cristol, Fourcade, and Seigneurn (1935).

than it is in alkaline, thus in the existence of this high concentration of hydrogen ions during the last few days of incubation, a mechanism is provided whereby this uric acid is left behind, encrusted on the remnants of the allantois, when the chick emerges from his shell

In the case of the amniotic fluid, the average pH is slightly above neutrality, but slightly below that of the allantoic fluid, on the 7th day. A maximum pH value is indicated on the 8th day, but this rise may not be significant. Generally speaking, during the period from the 7th to the 15th day of incubation, there occurs a gradual decrease in pH, by the 10th day, it has become slightly acid in its reaction. This change has probably been brought about by the increasing carbon dioxide production of the embryo with the attendant diffusion of this gas into the amniotic cavity, possibly this takes place by way of the allantoic circulation, but even more probably by direct diffusion from the embryo's tissues into the enveloping amniotic fluid. If this is so, the buffering capacity of the amniotic liquid is less than that found in the case of the allantoic

After the 15th day of incubation, the amniotic fluid becomes rapidly more alkaline, reaching a pH of 7.9 on the 19th day. These high values are interesting in that the allantoic fluid exhibits a decidedly acid reaction at this time. This demonstrates that the amnio-allantoic membrane cannot be permeable even to so small and readily diffusible an ion as hydrogen. Unquestionably, therefore, carbon dioxide could not be reaching the amniotic cavity by diffusion from the allantois during this period.

This change in the hydrogen ion concentration may possibly be linked with the absorption of certain materials from the amniotic fluid by the embryo, which is known to occur during this time. Protein materials from the albumen sac have been injected into this fluid (Hirota (1894)), only to disappear very shortly, according to the findings of Schenck (1932). This obviously results in a predominance of basic constituents, but again, the results of chemical analyses offer no satisfactory explanation as to their nature.

Comparisons between the specific conductance of these fluids (Walker (1943)) and their hydrogen ion concentration reveal certain interesting general relationships. In the case of the allantoic liquid, both specific conductance and pH are decreasing during the last few days of the period investigated. This decrease in the ionic strength of this fluid apparently does not involve a decrease in the number of hydrogen ions present in solution. The same correlation may be observed in the minima occurring for both properties on the 15th day in the case of the amniotic fluid. The high pH values for this latter fluid occurring on the 19th day correspond to fairly high conductance values, but the highest conductance values occur during a period in which pH is quite close to neutrality.

The investigation of changes in the pH of these two fluids has been studied previously by other workers. The present study, made with more advanced

technique, should be compared critically with these earlier results. In Fig 4, certain of these data have been plotted together with the results of the present series of experiments.

Of these earlier data, the two most important sets are those of Aggazzotti (1913) and of Yamada (1933). The former has for a long time been credited with reliability, as for example by Needham (1931*b*). The data of Buytendijk and Woerdeman (1927) are incomplete, and as seen in Fig 4 run slightly higher than, but close to, those of Yamada and the present data. Gueylard and Portier (1925), although credited by Needham (1931*b*)<sup>2</sup> with observations on the allantoic fluid, actually measured the pH of various mixtures of both amniotic and allantoic fluids, their results have little bearing on the present situation. Abe's observation (1927) that the pH of the amniotic fluid is constant at 7.1 throughout the course of incubation indicates the use of an inadequate method of measurement. The values obtained by Rubinstein (1932) for the amniotic fluid show too great a variation to be reliable.

One of the most striking features of the present data, however, is the confirmation afforded Yamada's results. The curve for the allantoic fluid is practically identical with that given by the Japanese worker, the fluctuations which do appear being of a very minor nature. As for the amniotic fluid, although the data are slightly higher than those obtained by Yamada, the agreement is nevertheless quite good. His determinations were carried only as far as the 17th day, but the upward trend shown by the present data is certainly indicated.

In contrast to these two sets of data stand the results obtained by Aggazzotti, who claims to have found the existence of extremely acid conditions within the amniotic fluid during the latter portion of the incubation period. pH values as low as 4.7 are reported on the 18th to 19th days. In fact, Aggazzotti's results are consistently lower than the present data and those of Yamada over practically the entire range of development. Aggazzotti does not give details of his method of pH determinations but merely states that the values were determined electrometrically. The fact that he confirmed his electrometric pH determinations by means of determinations of the titrable acidity gives more weight to his figures and therefore only accentuates this discrepancy.

However, although his physical measurements seem to have been carried out with reasonable exactitude there is reason to question his methods of obtaining samples of the fluids. For example, in connection with the allantoic fluid, he states that by the 8th or 9th day, he was no longer able to obtain pure samples of this fluid because of contamination by yolk. No such difficulties were encountered in the course of the present investigations, nor have they been reported by others who have worked with this material. Moreover, in

<sup>2</sup> Needham, J. *Chemical embryology* London, Cambridge University Press, 1931*b*, p. 57.

than it is in alkaline, thus in the existence of this high concentration during the last few days of incubation, a mechanism by which this uric acid is left behind, encrusted on the remnants of the shell, so that the chick emerges from his shell.

In the case of the amniotic fluid, the average pH is slightly below that of the allantoic fluid, on the 7th day the pH value is indicated on the 8th day, but this rise may be generally speaking, during the period from the 7th to the 10th day there occurs a gradual decrease in pH, by the 10th day the acid in its reaction. This change has probably been due to increasing carbon dioxide production of the embryo and its diffusion of this gas into the amniotic cavity, possibly through the allantoic circulation, but even more probably by the diffusion of the embryo's tissues into the enveloping amniotic fluid. The capacity of the amniotic liquid is less than that found in the allantoic fluid.

After the 15th day of incubation, the amniotic fluid becomes alkaline, reaching a pH of 7.9 on the 19th day. This change is due to the fact that the allantoic fluid exhibits a decidedly alkaline reaction. This demonstrates that the amnio-allantoic membrane is even to so small and readily diffusible an ion as  $\text{CO}_2$ , and therefore, carbon dioxide could not be reaching the embryo from the allantois during this period.

This change in the hydrogen ion concentration is due to the absorption of certain materials from the allantois, which is known to occur during this time. Proteins and amino acids have been injected into this fluid (Hirota (1931)). Shortly, according to the findings of Schenck (1932), there is a predominance of basic constituents, but the analyses offer no satisfactory explanation as to the cause of this change.

Comparisons between the specific conductance and the hydrogen ion concentration reveal interesting relationships. In the case of the allantoic fluid, the pH is decreasing during the last few days of incubation, and the decrease in the ionic strength of this fluid appears to be due to a decrease in the number of hydrogen ions present in the fluid. This may be observed in the minima occurring for the conductance in the case of the amniotic fluid. The minima occurring on the 19th day correspond to the highest conductance values occur during the approach to neutrality.

The investigation of changes in the pH of extra-embryonic fluids has been previously by other workers. The present work is a continuation of the work of

2 Changes in this property have been related to changes in the chemical composition of these two fluids.

3 The results of this investigation have been compared with those obtained by other workers. Excellent confirmation has been afforded the work of Yamada, whereas the work of Aggazzotti, which has long been accepted, is shown to be in error

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connection with the amniotic liquid, he describes it as quite clear and colorless, a description which does not agree with that of Yamada nor with observations made in the course of the present investigation. Yamada describes the amniotic fluid of 12 day embryos as slightly turbid, and at the end of the 2nd week of incubation it becomes, in addition, pale yellow in color. He further notes that it becomes very viscous at about this time. His observations have been afforded complete confirmation throughout the present investigation. Aggazzotti, strangely enough, fails to note any of these changes in the appearance of this fluid, a fact which would seem to indicate that he was not dealing with samples of the amniotic fluid at all.

As the result of what is apparently a *a priori* reasoning, Aggazzotti claimed that the embryonic urine was acid in reaction. Since his data show that the allantoic fluid is alkaline in all stages studied and that the amniotic first becomes acid on the 11th day, he has drawn the following conclusions: (1) the mesonephros is not functional, and (2) the metanephric secretions are deposited in the amnion. Fiske and Boyden (1926), however, have pointed out that it is impossible to predict, *a priori*, what the reaction of the embryonic excreta would be.

Aggazzotti then proceeded to attempt to explain how this embryonic urine could become deposited in the amniotic cavity. Contrary to the earlier work of Gasser (1880) on the development of the cloacal opening, he holds that, from about the 11th day on, the urinary secretions of the embryo pass out through some "physiological connection" between the cloaca and the amniotic cavity at the region of the Bursa of Fabricius. According to Lillie (1930),<sup>3</sup> this is without morphological basis. The experiments of Hanan (1925), (1927) on the excretion of trypan blue by the mesonephros of the developing chick show that physiological evidence is likewise lacking. Thirdly, chemical analyses of the amniotic fluid fail to reveal any appreciable amounts of uric acid at any time during incubation.

In view of these criticisms of Aggazzotti's interpretations, and the possibility that he was not dealing with pure samples of amniotic fluid, and in view of the excellent agreement between the present data and those of Yamada in spite of differences in technique employed, the latter sets of values should be considered as representing the hydrogen ion concentration of these extra-embryonic fluids, and the data of Aggazzotti should be discarded.

#### SUMMARY

1 The hydrogen ion concentration of the allantoic and amniotic fluids of the developing chick has been determined over the period of incubation between the 7th and 19th days using the glass electrode technique.

<sup>3</sup> Lillie, F. R., *The development of the chick*, New York, Henry Holt, 2nd edition, revised, 1930, pp. 314 ff.

2. Changes in this property have been related to changes in the chemical composition of these two fluids

3 The results of this investigation have been compared with those obtained by other workers Excellent confirmation has been afforded the work of Yamada, whereas the work of Aggazzotti, which has long been accepted, is shown to be in error

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# NATIVE AND REGENERATED BOVINE ALBUMIN

## I. PREPARATION AND PHYSICOCHEMICAL PROPERTIES\*†

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Investigation of the effect of denaturation on certain chemical, physicochemical, and biological properties of proteins has proved a valuable approach in the study of protein structure. Further advances have been made by a study of the reversal of the process of denaturation and by comparative measurements of the properties of a protein in the native and regenerated<sup>1</sup> states. Recently, evidence has been given to show that the denaturation of horse serum albumin (2) and of pseudoglobulin (3) by urea and guanidine hydrochloride are essentially irreversible processes (4-6) although the regenerated proteins simulate certain characteristic properties of the native materials from which they were derived.

Investigation of the immunological properties of native and regenerated horse serum albumin (7, 8) led to the discovery that the two proteins were immunologically equivalent, but that the regenerated albumin exhibited a greatly reduced antigenic activity. Since, however, regeneration did not produce any significant permanent changes in size, shape, or electrochemical properties of the protein molecules, which would profoundly affect their osmotic activity, it was felt that the regeneration process presented a feasible approach to the problem of minimizing undesirable reactions commonly occurring when foreign proteins are administered parenterally for therapeutic purposes.

The studies on the regeneration of horse serum albumin have now been extended to bovine albumin. Greater availability and the naturally milder antigenicity induced the selection of this protein which has already been suggested by some as a source of plasma substitutes.<sup>2</sup>

The present paper describes the preparation and physicochemical proper

\* This work was carried out with the aid of grants from The Rockefeller Foundation, the Lederle Laboratories Inc. and the Duke University Research Council.

† A preliminary report of this investigation has already been published (1).

<sup>1</sup> "Regeneration" of a protein refers to the reversal of the process applied for denaturation and isolation of that fraction most closely resembling the native protein in chemical and physicochemical properties.

<sup>2</sup> The literature on this subject has been amply reviewed by Henry (9) Cohn *et al*, (10) and Janeway (11).

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ammonium sulfate, adjusted to pH 6.4, was added, dropwise, until a final concentration of 2.1 M was reached. The precipitate was allowed to settle over a period of 36 hours at 4° C. The supernatant fluid was siphoned off and both supernatant and residue filtered on a Buchner funnel. Under these conditions, a readily filtrable granular precipitate is obtained.

The combined filtrate and supernatant solution were adjusted to the isoelectric point of serum albumin, (pH 4.7) and the solution (now about 7 liters in volume) was slowly brought to a final concentration of 2.6 M at room temperature,<sup>5</sup> by dropwise addition of 4 M ammonium sulfate under continuous stirring. After the precipitate had settled overnight the supernatant liquor was siphoned off and the granular precipitate in the residue filtered on a Buchner funnel. An average yield of about 95 gm. of moist albumin filter cake was obtained per liter of serum. The supernatant solution gave a slight test for protein.

Albumin precipitates obtained in this manner from various batches of beef serum were combined, dissolved in a minimum quantity of distilled water, and dialyzed in the cold against running tap water and distilled water until free of ammonia. The suspension was next adjusted to pH 5.0, and, after standing overnight in the ice box, the precipitated euglobulins were centrifuged off the clear supernatant being set aside for reprecipitation of the albumin. The yield was about 18 gm. of protein per liter of serum.

Reprecipitation was carried out at room temperature. The solution was adjusted to pH 4.7, and the salt concentration raised to 2.4 M, with respect to ammonium sulfate by a combination of gradual addition of solid salt and of a 4 M solution of ammonium sulfate. A bulky precipitate was collected; the supernatant solution, containing only small amounts of protein, was discarded. After washing the precipitate on the Buchner funnel with a solution of 2.4 M ammonium sulfate, it was dissolved in a minimum quantity of water and dialyzed as before. A slight amount of globulin was removed by isoelectric precipitation at pH 5.0. The clarified albumin solution was preserved in the frozen state. Yield 12 gm. protein per liter of serum.

The product from the second precipitation was essentially homogeneous in electrophoresis and in diffusion, containing only approximately 4 per cent total globulins as revealed by analysis of the electrophoretic patterns (*cf.* Table III). The small decrease in the relative amount of globulin impurities obtained upon the second precipitation did not warrant further purification in view of practical considerations already stated.

Several attempts at crystallization of the amorphous albumin preparation were undertaken, involving variations of pH, temperature, and salt concentration. In all cases non-crystalline granular precipitates were obtained, although in some instances intense schlieren effects indicative of incipient crystal formation, were observed after prolonged stirring.

The above procedure for the preparation of bovine albumin was adopted after some experimentation. In early work an initial step involving alcohol precipitation in the cold (16) by the rotating membrane method (17) was included. These batches

<sup>5</sup> Occasionally, it was found profitable to raise the salt concentration to 2.8 M.

ties of native and regenerated whole albumin whereas the immunological properties of these proteins will be the subject of a subsequent publication (12)

### EXPERIMENTAL

#### *I Methods of Preparation*

*Native Whole Bovine Albumin*—In the preparation of whole bovine albumin existing methods of salt fractionation were modified to give a procedure adaptable to rapid large scale isolation of reasonably pure products. Previous work (13, 14) had already indicated that simple salt precipitation was an insufficient means for the preparation of electrophoretically homogeneous bovine albumin.<sup>3</sup> Rather than devise elaborate methods for further purification, it was proposed to rely for the elimination of globulin impurities on their relatively greater susceptibility to irreversible denaturation by urea or guanidine hydrochloride, since it had already been demonstrated that up to 85 per cent of serum globulins will precipitate upon regeneration (3), as compared with only 15 per cent for serum albumin (2).

Essentially homogeneous albumin preparations were obtained by low temperature precipitation of globulins with ammonium sulfate (2.1 M) at pH 6.4, followed by room temperature precipitation of the albumin at the pH of its isoelectric point (pH 4.7), at 2.6 M ammonium sulfate (13). Euglobulin impurities were removed by isoelectric precipitation from salt-free albumin solutions at pH 5.0. Two precipitations of albumin were found sufficient to yield a satisfactory product.

In this method, advantage is taken of the difference in the respective temperature coefficients of solubility of the bovine serum proteins. It was found that bovine albumin, like horse, but unlike human albumin (15), has a negative temperature coefficient of solubility, whereas the solubility of the horse and bovine globulins seems to be governed by a positive temperature coefficient. The sharpness of separation of the serum proteins is thus materially aided by precipitation of the globulins in the cold.

The preparation was followed throughout by identification of the products by their electrophoretic mobility.

5 liters of beef serum,<sup>4</sup> clarified by passing through a Chardin filter, No. 5254, were adjusted to pH 6.4 with 2 N acetic acid. Slow precipitation was effected in a 4° C. bath, under mechanical stirring, by intermittent addition of sufficient solid ammonium sulfate to give a final concentration of about 1 M. A solution of 4 M

<sup>3</sup> Although the crystallization of highly purified preparations of bovine albumin has been reported (10), details of the procedure remain unpublished.

<sup>4</sup> We are indebted to Drs. W. G. Malcolm and B. M. Lyon of the Lederle Laboratories, Inc., for a generous supply of beef serum.

ammonium sulfate, adjusted to pH 6.4, was added, dropwise, until a final concentration of 2.1 M was reached. The precipitate was allowed to settle over a period of 36 hours at 4° C. The supernatant fluid was siphoned off and both supernatant and residue filtered on a Buchner funnel. Under these conditions, a readily filtrable granular precipitate is obtained.

The combined filtrate and supernatant solution were adjusted to the isoelectric point of serum albumin, (pH 4.7), and the solution (now about 7 liters in volume) was slowly brought to a final concentration of 2.6 M at room temperature,<sup>5</sup> by dropwise addition of 4 M ammonium sulfate under continuous stirring. After the precipitate had settled overnight, the supernatant liquor was siphoned off and the granular precipitate in the residue filtered on a Buchner funnel; an average yield of about 95 gm. of moist albumin filter cake was obtained per liter of serum. The supernatant solution gave a slight test for protein.

Albumin precipitates obtained in this manner from various batches of beef serum, were combined, dissolved in a minimum quantity of distilled water, and dialyzed in the cold against running tap water and distilled water until free of ammonia. The suspension was next adjusted to pH 5.0 and, after standing overnight in the ice box, the precipitated euglobulins were centrifuged off, the clear supernatant being set aside for reprecipitation of the albumin. The yield was about 18 gm. of protein per liter of serum.

Reprecipitation was carried out at room temperature. The solution was adjusted to pH 4.7, and the salt concentration raised to 2.4 M, with respect to ammonium sulfate by a combination of gradual addition of solid salt and of a 4 M solution of ammonium sulfate. A bulky precipitate was collected; the supernatant solution, containing only small amounts of protein, was discarded. After washing the precipitate on the Buchner funnel with a solution of 2.4 M ammonium sulfate, it was dissolved in a minimum quantity of water and dialyzed as before. A slight amount of globulin was removed by isoelectric precipitation at pH 5.0. The clarified albumin solution was preserved in the frozen state. Yield: 12 gm. protein per liter of serum.

The product from the second precipitation was essentially homogeneous in electrophoresis and in diffusion, containing only approximately 4 per cent total globulins as revealed by analysis of the electrophoretic patterns (cf. Table III). The small decrease in the relative amount of globulin impurities obtained upon the second reprecipitation did not warrant further purification, in view of practical considerations already stated.

Several attempts at crystallization of the amorphous albumin preparation were undertaken involving variations of pH, temperature and salt concentration. In all cases non-crystalline granular precipitates were obtained although in some instances intense schlieren effects, indicative of incipient crystal formation, were observed after prolonged stirring.

The above procedure for the preparation of bovine albumin was adopted after some experimentation. In early work an initial step involving alcohol precipitation (the cold (16) by the rotating membrane method (17) was included. These batches

<sup>5</sup> Occasionally, it was found profitable to raise the salt concentration to 2.8 M.



were pooled with others that had undergone initial salt precipitation, and the whole material purified by the method described above. The electrophoretic patterns were superimposable with those of lots prepared in later work. The purified product obtained on pooling was used in most of the experiments reported in this paper.

*Regenerated Whole Bovine Albumin*—Regenerated bovine albumin was prepared by the procedure already described for horse serum albumin (2). Bovine albumin dissolved in an 8 M solution of urea or guanidine hydrochloride was allowed to stand at room temperature for 24 hours and then dialyzed in the cold against running tap water and distilled water, until freed of the denaturing agent. The solution was then adjusted to pH 5.25 (with 2 N acetic acid), subsequent exposure for 30 minutes to a temperature of 41° C in a water bath resulted in the precipitation of an insoluble fraction. The precipitate was centrifuged off, and the supernatant solution of regenerated protein was made 1:10,000 with merthiolate and stored in the cold in a toluene atmosphere. In order to simplify the preparation, reprecipitation of the regenerated albumin was omitted.

Urea (Merck) and guanidine hydrochloride (Eastman) used in this investigation, were purified products recrystallized by methods already described (2). Concentrated aqueous solutions had a pH of 6.8 to 7.0.

## *II Chemical and Physical Properties of Native and Regenerated Bovine Albumin*

### *1 Nitrogen and Carbohydrate Content*

Dry weight of the protein was determined by drying at 110° C for 24 hours, and ash content by ashing in a muffle furnace. Nitrogen analyses were made by the semi-micro-Kjeldahl method. The results of triplicate determinations were in good agreement.

On a dry weight basis, the ash content of native bovine albumin, prepared as above, was 0.2 per cent and the nitrogen content 15.2 per cent.

Carbohydrate determinations were made in triplicate by the orcinol method of Sørensen and Haugaard (18), with blank correction for the color developed by sulfuric acid and protein. Readings were made on an Evelyn photoelectric colorimeter (filters 520 and 540), with reference to an equal mixture of dextrose and mannose (0.1 mg sugar per cc) as a standard.

The carbohydrate contents of native, guanidine hydrochloride-regenerated, and urea-regenerated bovine albumin were 0.38, 0.39, and 0.44 per cent respectively. The results of these analyses indicate that no carbohydrate is split off upon denaturation and regeneration, a finding in agreement with Neuberger's observation (19) of an unchanged carbohydrate content of egg albumin after denaturation by acid or heat.

## 2 Molecular Weight and Shape

## Methods

*Viscosity*—Measurements were carried out at  $25^{\circ} \pm 0.01^{\circ}$  C. with the modified Ostwald viscometers described previously (20)

*Diffusion*—Measurements were performed with the refractometric scale method

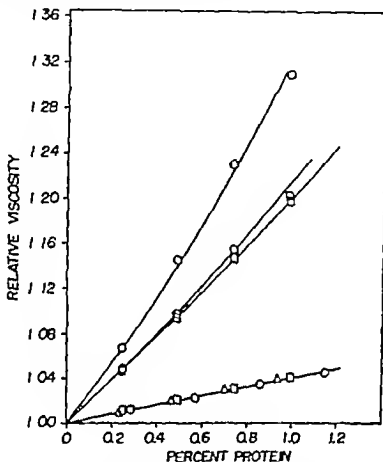


FIG 1 Relative viscosities of bovine serum albumin plotted against the protein concentration in weight per cent. In order of decreasing slopes the curves refer to respectively denatured serum albumin in 7.42  $\mu$  guanidine hydrochloride (O), in 3.96  $\mu$  guanidine hydrochloride (O) and in 8  $\mu$  urea (□). The curve of the least slope refers to native bovine albumin ( $\Delta$ ), the same protein regenerated from 8  $\mu$  urea (□), and regenerated from 8  $\mu$  guanidine hydrochloride (O)

at  $25^{\circ} \pm 0.003^{\circ}$  C. and were evaluated by methods described in previous publications from this laboratory (21)

*Sedimentation Velocity*—Samples of whole bovine albumin of albumin regenerated from 8  $\mu$  urea, and from 8  $\mu$  guanidine hydrochloride, were sent to Dr Max A Lauffer of The Rockefeller Institute for Medical Research, Princeton, New Jersey, who very generously performed sedimentation velocity measurements with the technique commonly employed in that laboratory. The analyses were made in an 0.023  $\mu$  acetate buffer of pH 5.0 containing 0.2  $\mu$  NaCl in protein concentrations of 1.0 and 0.5 per cent, respectively. The same buffer was employed for diffusion and viscosity measurements on these materials

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when measurements were made in early stages of the diffusion process but in good agreement once diffusion had progressed. The origin of this discrepancy was attributed to optical difficulties inherent in the measurement of steep refractive index gradients by the schlieren method a condition leading to errors in evaluating the height of the maximum ordinate and to poor fitting of the experimental curve with the baseline. The latter effect results in a gross error in the calculation of the second moment in the standard deviation method. Calculation by the maximum height or maximum height-area methods likewise induces a magnification of experimental errors since these methods also involve the squaring of certain terms.

As a result, measurements were considered reliable only when made after the initial steep gradient had become sufficiently diffuse. Accordingly, quantities enclosed in parentheses in Table I were disregarded in the calculation of average values.

Recourse was also had to a method of calculation which avoids squaring of any term, yet considers the inflection point as well as the maximum ordinate and the area. Lamm (23) has given the derivation of equation 1 used for this calculation

$$D = \frac{A\mu F}{\sqrt{8\pi H_m l}} \quad (1)$$

In equation 1  $A$  is the area under the curve as determined by graphical integration  $\mu$  half the distance between the inflection points,  $H_m$  the maximum ordinate and  $F$  a correction factor for photographic magnification and the optical distances between camera, cell and scale

In calculation of the average diffusion constant a correction for the viscosity of the solvent was applied according to equation 2

$$D' = D\eta/\eta_0 \quad (2)$$

in which  $D$  is the average of the measured diffusion constants,  $\eta/\eta_0$  the measured relative viscosity of the solvent, and  $D'$  the corrected diffusion constant. However, previous experience (2) had already made suspect the strict validity of this equation in solutions of high viscosity. Accordingly in the calculation of the molecular weight of denatured bovine albumin in 8 M urea an empirical correction was also applied,  $D'$  being multiplied by the factor 1.1

**Molecular Kinetic Constants**—Apparent molecular shapes neglecting hydration,  $(b/a)$ , of the native, denatured, and regenerated bovine albumin were calculated from the limiting slopes of the viscosity curves by graphical solution of the Simha equation (24) (Table II). Dissymmetry constants  $(f/f_0)_v$  were evaluated from viscosity measurements. Molecular weights were calculated from viscosity and diffusion data  $(M_v)$ , and from diffusion and sedimentation data,  $(M_s)$ . Values for the molecular shape assuming 33 per cent hydration  $(b/a)_h$  are also listed in Table II.

### 3 Electrophoretic Properties

#### Method

The Tiselius electrophoresis apparatus equipped with the Philpot-Svensson optical system as already described (4) was used for electrophoretic analysis of fractions

## RESULTS

*Viscosity*—The denaturing action on bovine albumin of urea (8 M) and guanidine hydrochloride (7.42 M and 3.96 M) were compared with one another in the presence of phosphate buffer, pH 7.0, containing 0.0204 M  $\text{Na}_2\text{HPO}_4$ , 0.0130 M  $\text{NaH}_2\text{PO}_4$ , and 0.1260 M  $\text{NaCl}$ . Practical difficulties of weighing and

TABLE I

*Diffusion Constants\* of Native, Denatured, and Regenerated Bovine Serum Albumin†*

Time	D <sub>1</sub>	D <sub>2</sub>	D <sub>3</sub>	D <sub>4</sub>	Time	D <sub>1</sub>	D <sub>2</sub>	D <sub>3</sub>	D <sub>4</sub>
Seconds	10 <sup>-7</sup>	10 <sup>-7</sup>	10 <sup>-7</sup>	10 <sup>-7</sup>	Seconds	10 <sup>-7</sup>	10 <sup>-7</sup>	10 <sup>-7</sup>	10 <sup>-7</sup>
1 Native bovine serum albumin					3 Bovine albumin regenerated from 8 M urea				
47,640	6.95	(8.59)	7.45	7.43	39,240	6.47	(8.40)	7.16	7.03
79,980	6.93	7.21	7.07	7.23	79,860	6.66	6.97	6.83	6.97
103,080	7.26	6.84	7.00	7.37	106,380	6.67	6.78	6.81	6.97
Average	7.16 × 10 <sup>-7</sup> ± 0.21				Average	6.85 × 10 <sup>-7</sup> ± 0.20			
D'	7.36 × 10 <sup>-7</sup>				D'	7.04 × 10 <sup>-7</sup>			
2 Bovine albumin denatured in 8 M urea					4 Bovine albumin regenerated from 8 M guanidine hydrochloride				
83,280	2.13			2.09	39,600	6.51	(7.53)	6.88	6.92
128,400	2.24			2.20	71,400	6.31	6.69	6.40	6.56
166,440	2.23			2.24	93,600	6.23	6.27	6.25	6.44
Average	2.19 × 10 <sup>-7</sup> ± 0.06				Average	6.50 × 10 <sup>-7</sup> ± 0.24			
D'	3.86 × 10 <sup>-7</sup>				D'	6.68 × 10 <sup>-7</sup>			

\*  $D$  denotes the diffusion constant in square centimeters per second,  $D_1$ ,  $D_2$ , and  $D_3$  the diffusion constants calculated by the maximum height, standard deviation, and maximum height-area methods respectively,  $D_4$  the diffusion constant calculated according to equation 1, and  $D'$  the average diffusion constant corrected for the viscosity of the solvent with equation 2.

† Protein concentration of 0.7 per cent in all cases.

transfer precluded comparison of viscosities at strictly equimolar concentrations of the denaturing agents.

The relative viscosities were determined in protein concentrations varying between 0.2 and 1.2 per cent and are plotted in Fig. 1 against concentrations, expressed in weight per cent. The limiting slopes of the curves were determined from the intercept when  $\eta_{sp}/c$  was plotted against  $c$ , where  $\eta_{sp}$  is the specific viscosity and  $c$  the concentration (22).

*Diffusion*—The results of diffusion measurements are given in Table I.

In these experiments it was observed that the maximum height, standard deviation, and maximum height-area methods (21) gave results that were in poor agreement

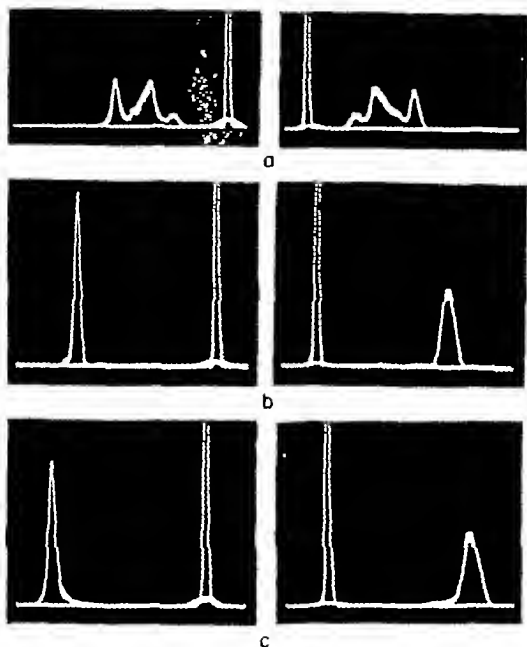


FIG. 2. Electrophoretic patterns of fractions of beef serum and of native and guanidine hydrochloride regenerated albumin. The left hand side of each picture represents the ascending boundary, the right hand side, the descending boundary. The sharp peaks near the center indicate the position of the starting boundaries. All runs were made at  $1^{\circ}\text{C}$ , pH 7.6, ionic strength 0.1, in a sodium veronal sodium chloride buffer. (a) Isoelectric euglobulin precipitate, 180 minutes migration at 5.70 volts per cm. (b) reprecipitated native bovine albumin, 240 minutes migration at 5.48 volts per cm. and (c) bovine albumin regenerated from 8 M guanidine hydrochloride, 246 minutes migration at 5.76 volts per cm. In the order of decreasing mobility the peaks in (a) represent respectively albumin,  $\alpha$ -globulin and  $\beta$ -globulin.

less than 10 per cent of the total protein (equivalent to less than 0.1 per cent protein concentration). Accordingly, the results of such determinations have been enclosed by parentheses in Table III.

Calculations were made from the descending boundary 100 per cent refers to the total area under the curve exclusive of that of the  $\epsilon$ -boundary

Representative electrophoretic diagrams of (a) the euglobulin fractions obtained by isoelectric separation from the first albumin precipitate, (b) purified native, and (c) guanidine hydrochloride-regenerated bovine albumin are reproduced in Fig 2

While the electrophoretic pattern reproduced in Fig 2 (a) reveals this isoelectric precipitate to consist largely of globulin, notably of the  $\alpha$ -component, it is surprising that as much as 25 per cent of this material is comprised of albumin which, by itself, is not susceptible to isoelectric precipitation

Electrophoretic analysis of the purified albumin in the native and regenerated state indicated the presence of less than 5 to 10 per cent globulin impurities With the materials regenerated by urea or guanidine hydrochloride, respectively, the globulins constituted about 4 per cent or less These figures compare favorably with those of Taylor and Keys (14) who report the presence of 5 to 7 per cent of a slow moving component, presumably globulin, in preparations of native bovine albumin obtained by either ammonium sulfate or methanol precipitation

Electrophoretic mobilities were used also to characterize native and regenerated bovine albumin All measurements were made under similar conditions at pH 7.6, ionic strength = 0.1 in the sodium veronal-sodium chloride buffer already described The mobilities given in Table IV are those calculated from the patterns of the descending boundary

No significant differences in mobility were observed between solutions of the first and second precipitate of a given preparation of bovine albumin or between various lots of purified native albumin Crystalline carbohydrate-free bovine albumin (obtained through the courtesy of the Department of Physical Chemistry, Harvard Medical School) was homogeneous at this pH and was characterized by essentially the same mobility as our preparations<sup>7</sup> For the sake of brevity this preparation will hereafter be referred to as "crystalalbumin" to distinguish it from our preparations of "whole albumin"

In agreement with results reported for crystalline carbohydrate-containing horse albumin (5), bovine albumin regenerated from 8 M urea demonstrates a slightly greater mobility at this pH However, under similar conditions albumin regenerated from 8 M guanidine hydrochloride is characterized by the same mobility as that of the native protein, a result confirmed by duplicated experiments Similar data for horse serum albumin regenerated from guanidine hydrochloride are unavailable

In summary, electrophoretic analysis indicates that native bovine albumin, and the same protein regenerated from 8 M urea or 8 M guanidine hydrochloride

<sup>7</sup> Carbohydrate analyses of crystalalbumin were made in this laboratory by the method already described (18)

are essentially identical in homogeneity and similar in mobility, with the exception of a slight increase in mobility observed for albumin regenerated from 8 M urea solutions.

TABLE IV

*Electrophoretic Mobilities\* of Native and Regenerated Bovine Albumin at pH 7.6 in NaV NaCl Buffer of 0.1 Ionic Strength†*

Preparation	Mobility	Remarks
	$\text{cm}^2 \text{ sec.}^{-1} \text{ volt}^{-1} \times 10^{-4}$	
BS II-VI, SA <sub>1</sub>	-6.29	First albumin precipitate of pooled sera II-VI.
BS II-VI, SA <sub>2</sub>	-6.22	Second albumin precipitate of above.
BS VII-VIII, SA <sub>1</sub>	-6.35	First albumin precipitate of pooled sera VII-VIII.
BS VII-VIII SA <sub>2</sub>	-6.39	Second albumin precipitate of above.
BS IX, SA <sub>1</sub>	-6.28	Second albumin precipitate of serum IX.
BS IX, SA <sub>2</sub>	-6.38	Precipitate of supernatant of above.
Mean mobility of whole native bovine albumin	$-6.32 \pm 0.07$	
Crystalalbumin	-6.42	Homogeneous
BS II-VI SA <sub>2</sub> regenerated from 8 M guanidine hydrochloride.	-6.29	Duplicate measurements
	-6.30	
BS II-VI, SA <sub>2</sub> regenerated from 8 M urea	-6.55	

\* Calculated from patterns of the descending boundary.

† Buffer composition as given in the text.

#### 4 Enzymatic Hydrolysis of Native and Regenerated Bovine Albumin

The rates of tryptic hydrolysis of whole native and regenerated bovine albumin were investigated in an attempt to detect differences in internal structure occurring upon denaturation and regeneration. A similar investigation of crystalline horse albumin has already been described (6).

##### Method

The conditions of these experiments and the methods involved were identical with those previously described (6) the only deviation being that in this study double quantities were used throughout in order to increase sensitivity. Pancreatin



X (Merck) was used as the source of tryptic activity. Solutions of substrate (100 mg) and enzyme (80 mg) dissolved in a 0.05 M phosphate buffer of pH 7.8 were incubated at 37° C. The progress of hydrolysis was measured by formal titrations of aliquots of the solution of enzyme and substrate.

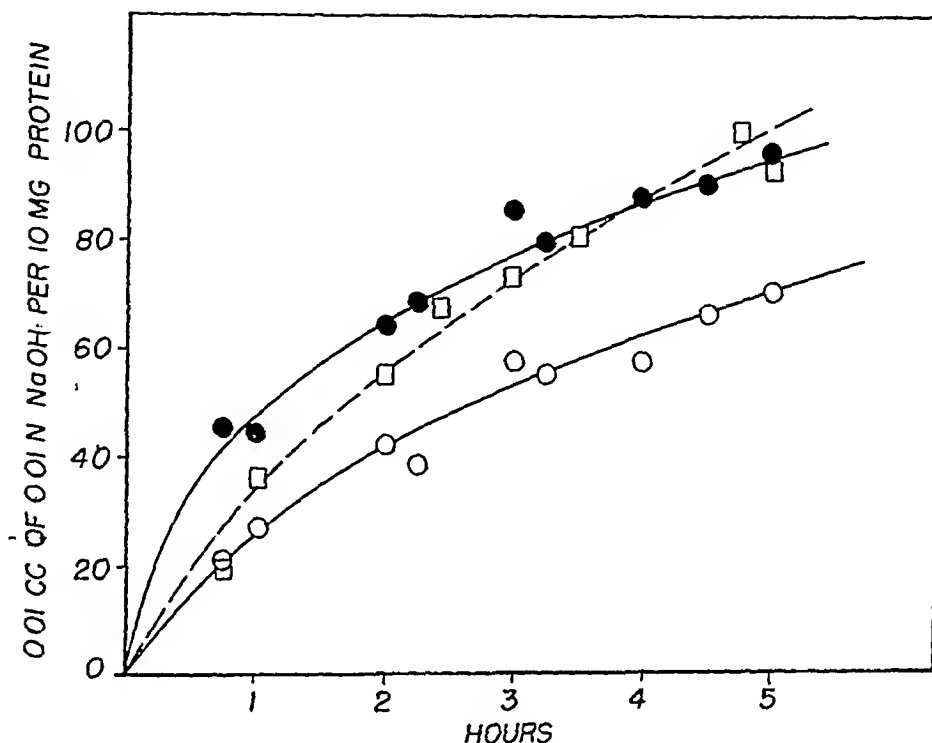


FIG. 3. Tryptic hydrolysis of native and regenerated bovine serum albumin. The lower curve (○) refers to whole native carbohydrate-containing albumin, the upper curve (●) to the same protein regenerated from 8 M guanidine hydrochloride, and the broken curve (□) to native, crystalline, carbohydrate-free albumin. In all cases, 100 mg of protein were subjected to hydrolysis by 80 mg of pancreatin X.

#### RESULTS

The results of duplicate determinations are given in Fig. 3, in which the amount of  $10^{-2}$  N NaOH required for the formal titration of 10 mg of substrate is plotted against the time of proteolysis.

As may be seen from Fig. 3, the rate of hydrolysis of the regenerated protein exceeded that of native albumin. The magnitude of the difference in the proteolytic rates of native and regenerated protein, as well as the extent of hydrolysis in each instance, is comparable to that observed for native and urea-regenerated horse serum albumin (6). By analogy, it may be inferred that the increased susceptibility of regenerated bovine albumin is an indication that this protein is actually in a denatured state.

The initial rate of hydrolysis of native bovine crystalalbumin (Fig 3) is intermediate between that of whole native albumin and of whole albumin regenerated from 8 M guanidine hydrochloride. However, at all times the proteolytic rate of native crystalalbumin is greater than that of native whole albumin and soon approaches that of whole albumin regenerated from 8 M guanidine hydrochloride.\*

### 5 *Distribution between Regenerated and Irreversibly Denatured Bovine Albumin* *Method*

The distribution between regenerated and irreversibly denatured protein was studied as a function of the concentration of urea and guanidine hydrochloride for both whole bovine albumin and crystalalbumin.

2 per cent solutions of the protein were allowed to stand at room temperature for about 24 hours in a given concentration of the denaturing agent. They were then dialyzed in the cold until free of urea or guanidine hydrochloride, dialysis often resulting in the precipitation of irreversibly denatured protein on the walls of the container. The soluble regenerated albumin was separated from the insoluble irreversibly denatured protein by the method of heating at 41° C. at pH 5.25 for 30 minutes, followed by centrifugation and washing of the precipitate (2).

Separation of irreversibly denatured and regenerated protein may also be effected by fractional precipitation with salt. Experiments previously reported for horse serum albumin (2) demonstrated both methods to yield the same quantitative distribution. In view of the rather wide limits of salt concentrations within which whole bovine albumin is precipitated from solution the heating method was chosen for this investigation.

Nitrogen analyses of aliquots of the supernatant solution were made in duplicate by the semi-micro-Kjeldahl method, precipitates were analyzed similarly.

### RESULTS

In Fig. 4, the per cent of irreversibly denatured protein is plotted as a function of the concentration of the denaturing agent. It may be observed that for whole albumin a recovery of 95 per cent of the original protein as regenerated albumin is obtained, independent of the concentration of guanidine hydrochloride above 4 M. The corresponding experiment involving whole albumin in urea was not undertaken because of the gratifying yield found for guanidine hydrochloride, and since previous experience with horse albumin had indicated

\* The corresponding experiment involving the tryptic digestion of regenerated bovine crystalalbumin has been performed, giving the result that the rate of hydrolysis of native crystalalbumin constantly was less than that of the same protein regenerated from 8 M guanidine hydrochloride. These experiments are subject to publication at a later date.

the quantitative distribution to approach the same level in higher concentrations of urea or guanidine hydrochloride (2)

With crystalbumin, a considerably higher yield of irreversibly denatured protein was observed than for the whole bovine albumin. The relation between concentration of urea, or guanidine hydrochloride, and per cent of

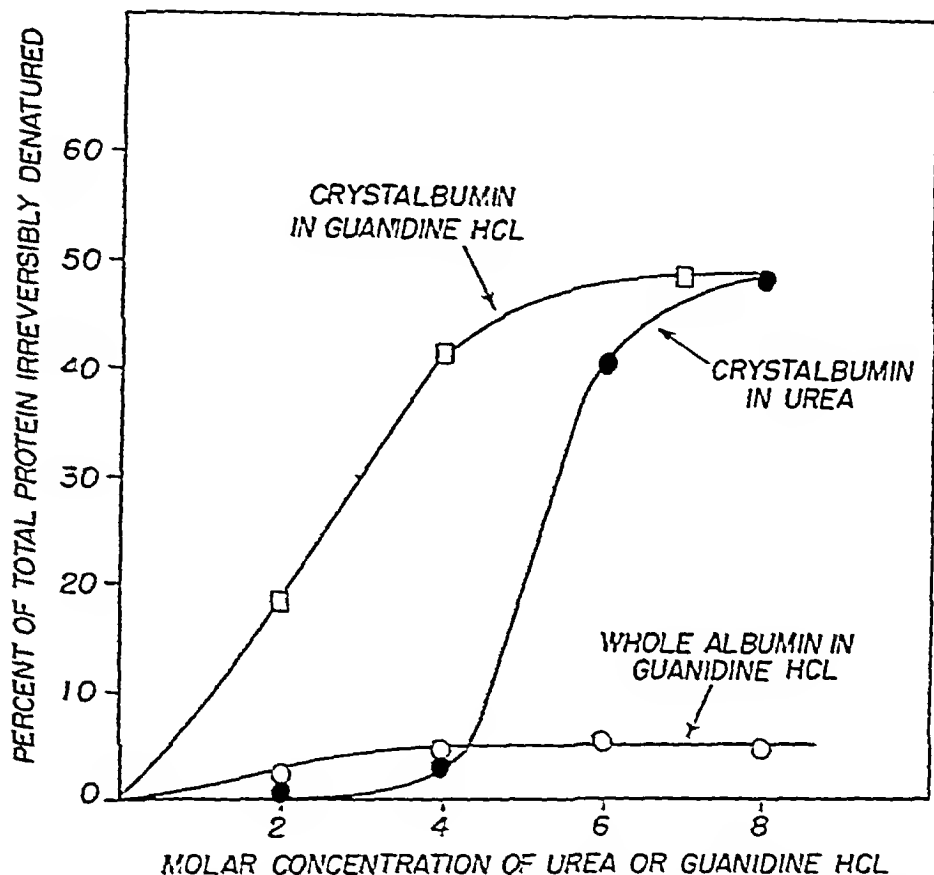


FIG 4 The fraction of total bovine serum albumin irreversibly denatured after dialysis, plotted against the molarity of urea or guanidine hydrochloride at which denaturation occurred

irreversibly denatured material is in qualitative accord with that obtained with crystalline horse serum albumin.

#### DISCUSSION

Modification of existing methods of salt fractionation has enabled the preparation of beef serum albumin which after reprecipitation, is about 95 per cent homogeneous electrophoretically, it appears homogeneous when studied by sedimentation and diffusion methods. The protein represents whole albumin,

and, in analogy with horse serum albumin (25, 26), probably contains components of varying carbohydrate content. This is also borne out by the fact that crystalline, carbohydrate-free bovine albumin has already been isolated (10).

In the method of preparation, advantage has been taken of the observation that bovine albumin, as well as horse albumin (27), exhibits a negative temperature coefficient of solubility in concentrated ammonium sulfate solutions. With either protein this property may readily be demonstrated by observation of the precipitate arising when a cold solution of the protein, already brought to the point of incipient turbidity with ammonium sulfate, is slowly allowed to attain room temperature.\* In this respect, horse and beef albumin appear to differ from human albumin, for which Kendall (15) reports a positive temperature coefficient of solubility under similar conditions.

The molecular kinetic constants of native whole bovine albumin, as determined by diffusion, sedimentation, and viscosity measurements, are similar to those found for horse serum albumin (2, 10). The molecular shape properties correspond to those of a prolate ellipsoid of revolution. The molecular weight, when calculated from diffusion and viscosity data, is  $65,000 \pm 6,000$ , and  $61,000 \pm 6,000$  when calculated from diffusion and sedimentation data. In both instances a value of 0.75 has been assumed for the partial specific volume.

The electrophoretic mobility of this protein at pH 7.6 is close to that of a mixture of fractions A and B of horse serum albumin, when measured under similar conditions (5).

Denaturation of bovine albumin by urea and guanidine hydrochloride follows the same pattern already described for horse albumin. The extent of denaturation was evaluated by a study of the distribution between irreversibly denatured, and regenerated components, and by measurements of the increase of the specific viscosities of the protein solutions. Both criteria indicated a greater denaturing power of guanidine hydrochloride as compared with equimolar concentrations of urea.

While gross changes in molecular *shape*, concurrent with denaturation, are apparent, evaluation of molecular *weights* in the presence of high concentrations of the denaturing agents is attended with some difficulty. As already noted, limitations in the diffusion method led to the introduction of empirical correction terms. While the calculated molecular weight of whole bovine albumin in 8 M urea solutions is considerably higher than that of the native protein,

\* With horse serum albumin, the formation and growth of crystals may actually be seen through the microscope by placing a drop of the cold solution on the slide. Crystallization starts instantaneously and is complete after about 5 minutes. Crystals formed by exposing the cold solution to room temperature redissolve when the suspension is chilled to 4° C. This reversible process of solution may be repeated several times.

examination of the diffusion curves by the method of successive analysis failed to indicate significant deviations from homogeneity, thereby excluding aggregation as a factor in raising the apparent molecular weight of the denatured protein. A similar situation has been experienced with horse serum albumin (2) although osmotic pressure measurements (28) indicate that the molecular weight of this protein remains unchanged upon denaturation by concentrated urea solutions. Determinations of the true molecular weight of urea-denatured bovine albumin must await enlistment of independent methods of investigation.<sup>10</sup>

The regenerated protein molecules bear a certain resemblance to the intact native unit. No changes in carbohydrate content occur during the denaturation-regeneration procedure, while the drastic changes in molecular shape resulting from denaturation become quantitatively reversed during regeneration. Within the limits of the resolving power of the present methods, native and regenerated whole bovine albumin reveal almost identical molecular weights, although a slight elevation has been found to result upon regeneration from concentrated solutions of guanidine hydrochloride.<sup>11</sup>

In some procedures for the modification of proteins, changes in the degree of homogeneity occur.<sup>12</sup> However, analysis by the methods of electrophoresis, diffusion, and sedimentation reveals that the present method not only preserves the homogeneity of the native protein but that, actually, the regenerated protein contains a slightly reduced amount of electrophoretic impurities.

In previous studies on horse serum albumin, electrophoretic mobility has been considered as a criterion for the reversibility of the denaturation process (5). While, in analogy with those studies, urea-regenerated bovine albumin revealed a slightly higher mobility than the native protein, no changes in mobility could be found following regeneration from guanidine hydrochloride. This difference in behavior does not invite elaborate explanation as long as analogous data on horse serum albumin remain unavailable.

Comparative measurements on the rates of tryptic hydrolysis have also been considered as sensitive criteria for the reversibility of the denaturation process. Here, bovine albumin follows closely the behavior previously established for horse serum albumin (6). To a considerable degree, the regenerated material is more susceptible to tryptic fission than the native material. This finding, together with the observation that irreversibly denatured, and regenerated horse serum albumin are equally susceptible to proteolysis, indicates the occurrence of irreversible changes in the structure of the bovine albumin con-

<sup>10</sup> Osmotic pressure measurements are being undertaken in this laboratory in the hope of solving this problem.

<sup>11</sup> This apparent increase in molecular weight may arise from an increase in the partial specific volume of the protein.

<sup>12</sup> Cf. reference (9), page 471.

comitant to the denaturation regeneration process. Denaturation of these proteins, under the conditions described, is therefore, irreversible.

The reversible denaturation and inactivation of crystalline proteolytic enzymes, studied by Northrop, Kunitz, and Herrnott (29), appears to be a somewhat different process. Under specified conditions of pH and temperature reversal could be achieved only if the proteins were exposed to the denaturing action for a short period of time. In the present studies, however, the serum proteins were exposed to a given concentration of the denaturing agent long enough for the denaturant to exert its full effects. Under these conditions, denaturation is irreversible. Similar considerations may apply (30) to the discrepancies between the present findings and those obtained by Anson and Mirsky (31) for the reversible denaturation of hemoglobin by sodium salicylate.

The bovine crystalalbumin used in this investigation represents a highly purified fraction of whole albumin (10), devoid of carbohydrate constituents. Although it resembles whole albumin in certain physicochemical properties, its molecular weight and electrophoretic mobility, differences in intrinsic structure may be revealed by factors significant in characterizing susceptibility to denaturation.

A larger proportion of crystalalbumin was irreversibly denatured by concentrated solutions of urea or guanidine hydrochloride than was found for whole bovine albumin. As indicated by Fig 4, the corresponding quantities are about 50 per cent and 5 per cent respectively. Unpublished data on hand indicate that the viscosity increase produced by 8 M urea solutions is greater for crystalalbumin than for whole albumin. Finally, crystalalbumin has been found to be more susceptible to tryptic hydrolysis than the protein from which it was derived (Fig 3). Hence, by these three criteria, crystalalbumin is more liable to denaturation and less susceptible to regeneration, than whole native bovine albumin.

Our thanks are due to Dr. Max A. Lauffer of the Rockefeller Institute for Medical Research, Princeton, New Jersey, for his generous cooperation in performing the sedimentation analyses, we are also indebted to Dr. J. W. Beard of the Department of Experimental Surgery, Duke University School of Medicine, for making available to us the Tiselius apparatus used in electrophoresis measurements and to Drs. E. J. Cohn and H. B. Vickery, Harvard Medical School for arranging for the supply of crystalline bovine albumin.

#### SUMMARY

1. Whole bovine albumin, homogeneous in diffusion and sedimentation, and essentially homogeneous in electrophoresis, has been prepared by a method involving ammonium sulfate precipitation of the globulins in the cold and of

the albumin at room temperature, isoelectric precipitation of the euglobulins, and reprecipitation of the albumin

2 The product has been characterized by chemical analysis and by viscosity, diffusion, sedimentation, and electrophoresis measurements. The carbohydrate content is 0.38 per cent, the nitrogen content, 15.2 per cent. The molecular shape approximates that of a prolate ellipsoid with an axial ratio of 3.1, assuming 33 per cent hydration, the average molecular weight is 65,000.

3 Bovine albumin is readily denatured by concentrated solutions of urea or guanidine hydrochloride, gross changes in molecular shape resulting.

4 Regeneration of bovine albumin denatured in solutions of 8 M urea or guanidine hydrochloride yields a material closely resembling the native in carbohydrate content, in molecular size and shape, and in electrophoretic properties. However, the regenerated protein differs from the native in susceptibility to tryptic digestion, and, in this respect, appears to be in a denatured state.

5 In 8 M solutions of guanidine hydrochloride a limiting yield of regenerated albumin equivalent to 95 per cent of the original protein is approached.

6 Bovine crystalalbumin, a crystalline carbohydrate-free fraction of the whole albumin, appears to be more susceptible to denaturation than whole bovine albumin.

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# NATIVE AND REGENERATED BOVINE ALBUMIN

## II. IMMUNOLOGICAL PROPERTIES\*†

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Previous experiments have shown (2) that regeneration<sup>1</sup> of urea-denatured horse serum albumin yielded a material which, in comparison with the native protein, exhibited a significantly lower ability to stimulate precipitins in the rabbit. The regenerated protein, however, was capable of precipitating to full titer the precipitins produced by immunization of rabbits with the native material.

Similar experiments have now been carried out on whole bovine albumin, a protein of recognized lower antigenic activity than horse albumin. The preparation and properties of the native and regenerated proteins have been described in the preceding paper of this series (3)

### EXPERIMENTAL

#### Methods

1 *Antigens*—The materials used for injection and for titrating the serum for the presence of antibodies were (1) native whole bovine albumin (2) albumin regenerated from 8 M urea solutions, and (3) albumin regenerated from 8 M solutions of guanidine hydrochloride (3)

2 *Immunization of Animals*—Each antigen was injected intravenously into twenty four rabbits of varying weights and breeds, each animal receiving six doses, given twice weekly over a 3 week period. To compare the effects of dosage each group of twenty four rabbits was divided into four groups of six each. The native and urea regenerated whole bovine albumin antigens were administered in total doses of 10 mg, 20 mg, 40 mg and 80 mg per kilo body weight, while due to a misunderstanding the corresponding doses of whole bovine albumin regenerated from guanidine hydrochloride, were 6 mg, 12 mg, 24 mg and 48 mg per kilo. For each group the first two doses were one-tenth of the total dose the last four injections each containing

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† A preliminary report has already been published (1)

<sup>1</sup> "Regeneration" of a protein refers to the reversal of the process applied for denaturation and isolation of that protein fraction most closely resembling the native protein in chemical and physicochemical properties.

one-fifth of the total dose. The rabbits were bled from the heart 7 days after the last injection.

**3 Precipitin Titration**—The serum dilution method, described by one of us (4) was used. 0.5 ml quantities of twofold dilutions of immune serum in saline, and 0.5 ml quantities of each antigen solution, containing 8  $\mu$ g of antigen per ml, were mixed, shaken, and examined for visible precipitation after standing for 2 hours at room temperature. The antigen concentration of 8  $\mu$ g per ml was selected after preliminary determinations had shown that a final concentration of 4  $\mu$ g per ml was the smallest amount of antigen capable of yielding a definitely visible precipitate under the conditions (Table I).

TABLE I

*Protocol of Preliminary Experiments to Determine the Smallest Amount of Antigen Capable of Yielding a Visible Precipitate in a Bovine Albumin Anti-Bovine Albumin Rabbit Serum System*

Rabbit serum No	Final serum dilution	Final concentration of antigen, $\mu$ g per ml										Antibody units per ml of undiluted serum
		1000	500	250	125	62.5	31.3	15.7	7.8	3.9	2.0	
3	1-4	+	+	+	+	+	+	+	+	+	—	256
6	1-4	+	+	+	+	+	+	+	+	+	—	128
9	1-8	$\pm$	+	+	+	+	+	+	+	+	—	512
10	1-4	+	+	+	+	+	+	+	+	+	—	256
18	1-8	+	+	+	+	+	+	+	+	+	—	1024
20	1-8	+	+	+	+	+	+	+	+	+	—	1024
21	1-8	+	+	+	+	+	+	+	+	+	—	512
23	1-64	—	$\pm$	+	+	+	+	+	+	+	—	2048
24	1-8	+	+	+	+	+	+	+	+	+	—	256

+, denotes visible precipitation, —, no precipitation, and  $\pm$ , doubtful reaction

Column 1 of Table I lists the serum employed and column 2 the final dilution of serum used in these constant antibody-varying antigen series. The following ten columns show the various final antigen concentrations, expressed in micrograms per milliliter. The table shows that the antigen dilution method yields constant endpoints which have no relationship to the precipitin titer as obtained by the serum dilution method (last column).

Similar titrations showed the concentration of 4  $\mu$ g per ml to be minimal also for observation of visible precipitation of the regenerated antigens with their respective homologous antisera.<sup>2</sup>

The accuracy of this method has already been demonstrated for the Pneumococcus, Type I SSS-antipneumococcus rabbit serum system (4). Its application to a protein-

<sup>2</sup> The results of precipitin titrations are expressed arbitrarily in terms of antigen concentration, following Marrack's suggestion (5) that the unit of antibody be defined as the amount reacting optimally with 1  $\mu$ g of antigen. The titration values given for each serum in Table I were obtained by subsequent titration by the serum dilution procedure described above.

antiprotein rabbit serum system was checked by independent titration of a rabbit serum containing antibodies for the horse serum albumin fraction A, prepared by the method of Kekwick (6). The equivalence point, determined by the quantitative method of Heidelberger and Kendall (7), was found to be attained with 1.23 mg of antigen, by Culbertson's method (8) a value of 1.0 mg was obtained. The highest serum dilution yielding a visible precipitate, when mixed with an equal volume of antigen containing 8  $\mu$ g per ml, was 1-128. In terms of final concentration, a serum dilution of 1-256 produced a precipitate with 4  $\mu$ g of antigen, indicating that the undiluted serum was capable of completely precipitating 1.024 mg of antigen (corresponding to 1024 antibody units).

4 *Anaphylaxis*—10 days after the last injection of antigen the rabbits were injected intravenously with homologous or heterologous antigens, in a dose of 100 mg per kilo body weight.

## RESULTS

1 *Anaphylaxis*—A few rabbits died during the immunization procedure, of the remainder only one rabbit (No. 23) showed any evidence of shock. However, it recovered completely within 15 minutes.

2 *Precipitin Titers*—The results of the precipitin titrations, obtained with the homologous antigens are summarized in Table II.

Column 1 of Table II refers to the nature of the antigen and the total immunizing dose, column 2 to the number of animals receiving this dose. The following columns give the number of antisera that were found to contain the number of antibody units listed in the top row of the table. Mean values for the antibody units, obtained in response to the administration of each immunizing dose, are listed in the last column of the table.

3 *Relation between the Immunizing Dose and Precipitin Content*—Inspection of Table II shows a marked variation in antibody titer within each group of antisera. Statistical analysis failed to reveal any significant correlation between the dose injected and the degree of precipitin response.

4 *Comparison of the Antigenicity of Native and Regenerated Whole Bovine Albumin*—Calculations of mean values for the antibody content of the first three groups of sera listed in Table II indicate that the antigenic activity of these three antigens decreases in the following order: native whole bovine albumin, urea regenerated albumin, and guanidine hydrochloride regenerated albumin. The corresponding antibody values, expressed in antibody units per milliliter of serum are, respectively, 383, 274, and 114. Computation of  $t$  values, however, reveals that the differences in antibody response to the native and urea regenerated proteins were not significant ( $p < 0.4 > 0.3$ ).<sup>\*</sup> In these

<sup>\*</sup>  $p$  is the probability that a given deviation from expectation (based upon hypothesis) shall occur by the action of pure chance. Thus a value of  $p = 0.2$  indicates that in 20 per cent of the total number of observations an event will occur by mere coincidence.

computations, all values were considered to carry equal weight, irrespective of the immunizing dose. A similar comparison of the responses to the native and guanidine hydrochloride-regenerated whole bovine albumin showed the differences in antigenic activity to be statistically significant ( $p < 0.02 > 0.01$ )

TABLE II

*Results of Precipitin Titrations of Rabbit Antisera to Native and Regenerated Bovine Albumins with their Respective Homologous Antigens*

Antigen* total dose	No of rabbits injected	Antibody units per ml										Mean titer (antibody units per ml )	
		<4	4	8	16	32	64	128	256	512	1024		2048
<i>mg</i>													
<i>N-10</i>	6	1			1	1	1	1	1				83
<i>N-20</i>	4								1	3			448
<i>N-40</i>	5					2	1		1		1		282
<i>N-80</i>	6								3	1	1	1	725
Total	21	1			1	3	2	1	6	4	2	1	383
<i>U-10</i>	5							3	2				175
<i>U-20</i>	4					1		1	1	1			232
<i>U-40</i>	6					1	1	2	2				144
<i>U-80</i>	6							2	3			1	555
Total	21					2	1	8	8	1		1	274
<i>G-6</i>	6		1			1	1	1	1	1			167
<i>G-12</i>	6					1	2	3					91
<i>G-24</i>	6			1		2	1	2					67
<i>G-48</i>	6						4		2				128
Total	24		1	1		4	8	6	3	1			114
<i>C-80</i>	6	1	3				2						26

\* N, denotes whole albumin, U, albumin regenerated from 8 M urea, G, albumin regenerated from 8 M guanidine hydrochloride, and C, native crystalalbumin

Although the mean antibody titer obtained in the series of antisera to guanidine hydrochloride-regenerated albumin may have been due to the smaller immunizing doses employed, comparison of the antigenicities of these two proteins appears to be justified in view of the observation that variation in antibody response to various doses of antigen lacked statistical significance.

Hence, regeneration from 8 M guanidine hydrochloride, but not from 8 M urea, resulted in a significant change in the antigenic property when compared to the antigenicity of the original whole bovine albumin.

5 *Antigenicity of Crystalalbumin*—A generous supply of crystalline, carbohydrate-free bovine albumin, obtained through the courtesy of the Department of Physical Chemistry, Harvard University Medical School, enabled us to compare the immunological properties of our preparations with those of a standardized preparation of a highly purified material (hereinafter referred to as crystalalbumin). Six rabbits were immunized with a standard dose of 80 mg of crystalalbumin, the highest immunizing dose employed in the preceding experiments. The antibody titers, given in the last row of Table II, show that the mean titer of these six rabbits was 26 antibody units per ml. The numerical difference between this titer and the mean antibody response elicited by the administration of 80 mg of native, whole bovine albumin (725 antibody units per kilo body weight), is statistically significant ( $p < 0.05 > 0.02$ ). However, no statistically significant difference could be found when the mean titer of antisera to crystalalbumin was compared with that of all antisera to whole bovine albumin regenerated from guanidine hydrochloride ( $p < 0.1 > 0.05$ ), although a numerical difference is apparent. In these computations, the difference in the size of the immunizing dose has been neglected, for reasons already stated in the preceding paragraph. It appears, therefore, that the loss in antigenicity resulting from further chemical purification of whole albumin, yielding crystalalbumin, is comparable to, if not greater than, that occasioned by regeneration of native whole bovine albumin from 8 M guanidine hydrochloride.

6 *Serological Specificity*—Each of the antisera to whole bovine albumin, in the native state (*N*) and after regeneration from urea (*U*) and guanidine hydrochloride (*G*), were titrated with the respective heterologous antigens. Antisera from rabbits that had received the highest immunizing dose of each antigen were titrated also with crystalalbumin (*C*). In addition, all anti-crystalalbumin rabbit sera were titrated with native whole bovine albumin. In all cases the heterologous and homologous titers were found to be identical. Immunological equivalence of the four antigens, i.e. native whole bovine albumin, albumin regenerated from urea and from guanidine hydrochloride, and crystalalbumin, appears, therefore, to be established although quantitative precipitin measurements may yet reveal small differences in the degree of cross-reaction.

#### DISCUSSION

Comparison of the immunological properties of native and regenerated whole bovine albumin reveals their mean antigenic activities to decrease numerically in the following order: (1) native albumin, (2) albumin regenerated from 8 M urea, and (3) albumin regenerated from 8 M guanidine hydrochloride. All antigens proved to be immunologically equivalent with each other as well as with native bovine crystalalbumin.

The decrease in antigenicity achieved by regeneration of whole albumin by

urea was statistically not significant. However, regeneration from guanidine hydrochloride proved effective in rendering albumin less antigenic, resulting in about a 70 per cent reduction in activity.

It may be of interest to compare these findings with those previously reported for native, and urea-regenerated horse serum albumin (2). While differences in species specificity between the albumin constituents of horse and beef serum, as well as the relatively lower degree of purity of whole bovine albumin, preclude a strict comparison, certain differences in behavior are apparent. With horse serum albumin, fraction A, more than 90 per cent of the initially high antigenic power could be eliminated by regeneration from urea, while the same treatment resulted only in a small reduction in antigenicity of the naturally less antigenic bovine albumin. The more powerful denaturing action of guanidine hydrochloride was required to achieve a significant reduction in the antigenicity of bovine albumin.

An explanation for the different behavior of these two proteins may possibly be found in two factors which have been considered influential in determining the antigenicity of a protein.

The first of these is the presence of carbohydrate constituents. It has been pointed out (2) that carbohydrate groups appear to confer a high degree of antigenic activity on bacteria as well as on certain serum proteins. The data on hand indicate a similar parallelism between carbohydrate content and antigenic power, the order of decreasing antigenicity being as follows: horse serum albumin, fraction A (1.95 per cent carbohydrate), whole bovine albumin (0.4 per cent carbohydrate), and bovine crystalline albumin (no carbohydrate). However, the degree of antigenic activity should not be attributed solely to the presence of carbohydrate residues, for, with both horse serum albumin and whole bovine albumin, the regenerated materials, in spite of unchanged carbohydrate content, exhibited a lower antigenicity than the native proteins from which they were derived.

A second factor to be considered in this connection is the contribution of the intact, specific configuration of the native protein molecule. Elsewhere it has been suggested (2) that processes bringing about partial or complete abolition of antigenic activity are those which break down the internal structure of the molecule. Different processes, when applied to a protein, may vary from each other in the extent to which they affect the internal configuration of the protein, guanidine hydrochloride, for instance, being more effective in some respects than urea (9, 10). Conversely, a given denaturing agent may affect different proteins to varying degrees (11). The experimental evidence presented previously (3, 9) suggests strongly that horse serum albumin, fraction A, is more extensively denatured by urea, and is less susceptible to regeneration, than is whole bovine albumin. The present immunological data are indeed indicative of the existence of a close relation between loss of antigenicity and the extent to which denaturation has occurred.

The low antigenic power of native bovine crystalalbumin may be ascribed to the combined influence of both of the aforementioned factors. This protein is devoid of carbohydrate, in addition, it is more susceptible to denaturation than either horse serum albumin or whole bovine albumin.

The fact that all four antigens proved to be immunologically equivalent, as revealed by the present method of titration, is very significant. It indicates that the observed differences in antigenic activity are probably not due to the production of different types of antibodies. It reveals that neither denaturation and regeneration, nor chemical purification produced decisive changes in the chemical structure of bovine albumin. Finally, it adds to the evidence that the carbohydrate residues in these large protein molecules, though a contributing factor in establishing the degree of antigenicity, play no significant rôle in the determination of serological specificity.

#### SUMMARY

1 The effects of regeneration of whole bovine albumin on antigenic activity and serological specificity were determined by precipitin measurements on rabbit antisera to (1) native whole albumin, (2) albumin regenerated from 8 M urea, and (3) albumin regenerated from 8 M guanidine hydrochloride.

2 While numerically the mean antibody response to these three antigens was found to decrease in the order named, only the difference in antigenic activity between native and guanidine hydrochloride-regenerated albumin was statistically significant. Native, crystalline, carbohydrate-free albumin (crystalbumin) was considerably less antigenic than native whole bovine albumin, its activity being comparable to, if not less than, that observed for guanidine hydrochloride-regenerated whole albumin.

3 All four antigens were immunologically equivalent.

4 The antigenic activity of these proteins is discussed in terms of protein structure and carbohydrate content.

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EFFECTS OF pH AND OF VARIOUS CONCENTRATIONS OF SODIUM,  
POTASSIUM, AND CALCIUM CHLORIDE ON MUSCULAR  
ACTIVITY OF THE ISOLATED CROP OF PERIPLANETA  
AMERICANA (ORTHOPTERA)\*

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In 1940, Griffiths and Tauber (13) published a preliminary account of attempts to devise a physiological salt solution for the isolated foregut of the American roach, *Periplaneta americana* L. Initially, they tested insect physiological solutions which were recommended by B  lar (2), Hobson (16), Yeager (25), TenCate (23), and Glaser (10). Only the solutions of B  lar and Hobson produced results which were at all satisfactory for the excised crop of the American roach. Secondly, amounts of the chlorides of sodium, potassium, and calcium were varied, and it was found that 14.6 gm. of NaCl, 0.45 gm. of KCl, 0.50 gm. of CaCl<sub>2</sub>, and 0.19 gm. of NaHCO<sub>3</sub> in 1 liter of water gave the best results among many mixtures tested, when contractions of the cockroach foregut were used as a criterion of adequacy.

The foregut of the American roach, used for these tests, may be divided into several more or less distinct regions: an esophagus, a crop or ingluvies, and a gizzard or proventriculus. The crop is composed of three layers of tissue (Fig. 1). The innermost layer is the cuticula, which, in section, appears to be of varying thickness, to have a roughened inner margin, and to be lined as though it were stratified. The middle is composed of a single layer of large epithelial cells. Muscle tissue surrounds the outside of the crop. Irregularly placed longitudinal fibers lie inside the encircling transverse muscles. All fibers are distinctly striated, with well defined sarcomeres. The activity of these muscle fibers was studied in the present investigation. Only those contractions which resulted in change in length of the ingluvial region, and hence in movement of the attached lever were recorded in the work described here. Neither simple peristalsis, anti-peristalsis, nor proventricular activity appeared to influence the action of the lever.

#### Method

The apparatus used for recording muscular activity of the crop in various salt mixtures was the same as that previously described by Griffiths and Tauber (13). The

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method involved use of a glass tube through which the solution to be tested was passed, and in which the crop was suspended from the recording lever. This lever was a capillary glass tube so arranged that actual amplitude of movement was magnified seven times as it was recorded upon a slowly revolving kymograph drum which made one revolution in 16 hours. Solutions were all aerated with oxygen. All work was done at room temperature, usually between 22 and 26°C.

Test solutions were made up in distilled water redistilled from glass. Various mixtures of chemically pure sodium chloride, potassium chloride, sodium bicarbonate, calcium chloride, sodium dihydrogen phosphate, hydrochloric acid, and sodium hydroxide were used in the experimental solutions. Concentrated stock solutions

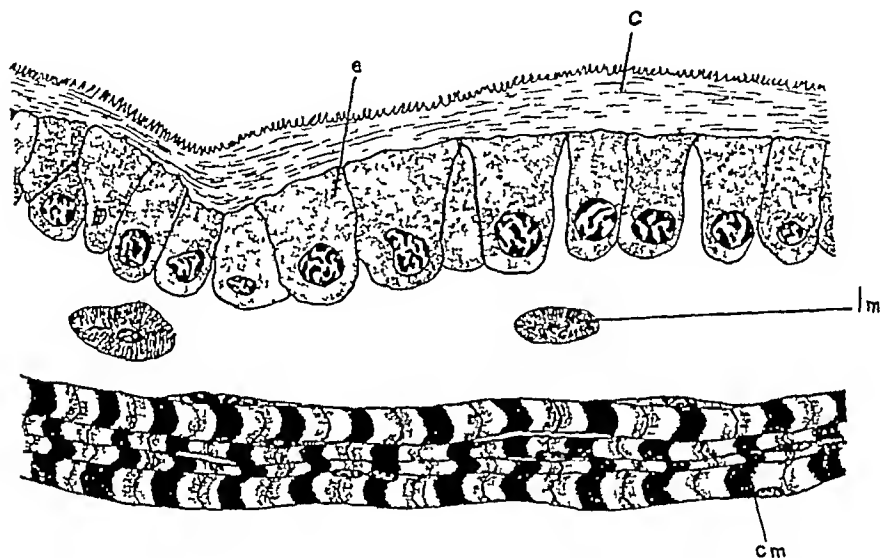


FIG 1 Drawing of portion of cross-section of crop from *Periplaneta americana*  
*c* = cuticula, *cm* = circular muscle, *e* = epithelium, *lm* = longitudinal muscle

were made of each of the above chemicals to facilitate preparation of dilutions to be tested.

Insects used in these investigations were adult specimens of the American cockroach, *Periplaneta americana* L. Only healthy individuals, between the ages of 15 and 100 days, were used. The roaches had all been laboratory-reared, at room temperature, on a diet of whole wheat bread, banana, raw beef steak, and Pabulum.

Ten foreguts, five from male roaches and five from females, were tested in each solution. Experimental animals were usually starved for 48 hours and then were given access to a mixture of ground starch and carmine which had been dampened with water. This colored food was used to determine food intake of specimens used for experimentation on the following day.

Each isolated crop preparation was allowed to run until failure to maintain spontaneous contractions terminated the experiment. Since the speed of the kymograph drum was known, duration of activity could be calculated in hours. Every 20 min-

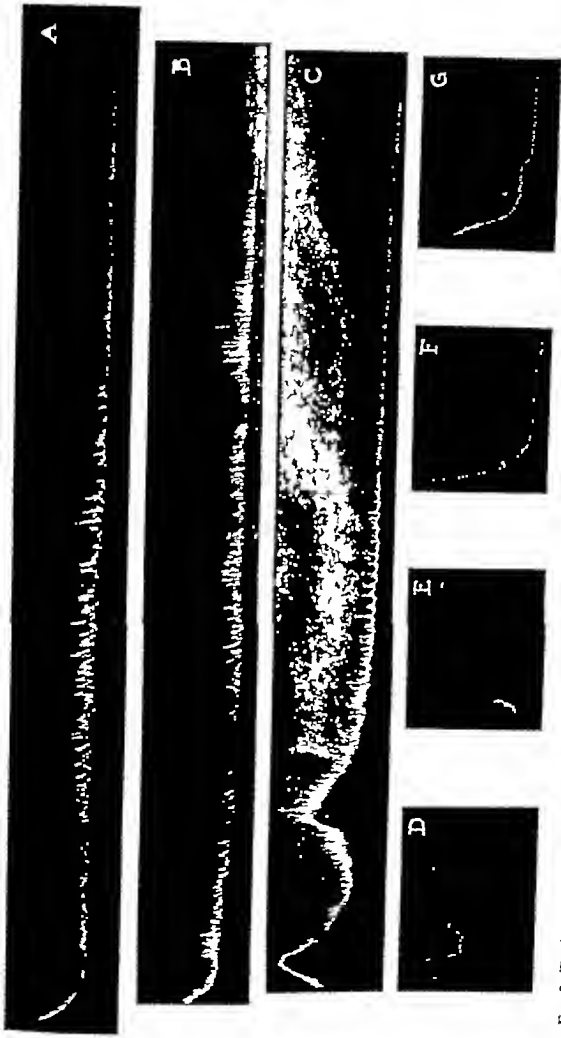


FIG. 2. Typical kymograph records of contractions of isolated American roach foreguts in salt solutions at pH of 7.8

A record from male in 1.4 per cent NaCl, 0.02 per cent KCl, 0.04 per cent  $\text{CaCl}_2$ ; B male 1.4 per cent NaCl, 0.02 per cent KCl, 0.02 per cent  $\text{CaCl}_2$ ; C female 1.0 per cent NaCl, 0.02 per cent KCl, 0.02 per cent  $\text{CaCl}_2$ ; D female 1.0 per cent NaCl, 0.02 per cent  $\text{CaCl}_2$ ; E male 1.0 per cent NaCl, 0.02 per cent KCl, 0.02 per cent  $\text{CaCl}_2$ ; F female 1.0 per cent NaCl, 0.02 per cent KCl, 0.02 per cent  $\text{CaCl}_2$ ; G female 1.0 per cent NaCl, 0.02 per cent KCl, 0.02 per cent  $\text{CaCl}_2$ .



tion with increased NaCl concentrations. The seven KCl/CaCl<sub>2</sub> ratios were within the range of 0.5 to 1.0.

Examination of activity products in Table I shows that satisfactory activity did not result in absence of either KCl, or CaCl<sub>2</sub>, or both. In mixtures containing KCl, but no CaCl<sub>2</sub>, the crops registered an initial loss of tone and stopped activity in a state of relaxation (Fig. 2, G). This phenomenon is in accord with the generally recognized depression effect of potassium upon all types of muscle tissue.

When CaCl<sub>2</sub> was present, but KCl was lacking, results were not so uniform. In slightly more than half the cases, the crop exhibited a sharp initial increase in tone and contractions ceased with the foregut in a state of contraction (Fig. 2, D and E). It appears that CaCl<sub>2</sub> is responsible for this increase in tonus,

TABLE I

*Average Activity Products for Solutions Containing 1.0 per cent Sodium Chloride, but in Which KCl/CaCl<sub>2</sub> Ratios Were Varied*

		KCl, gm. per liter				
		0.0	0.2	0.4	0.6	0.8
		cm.-hrs	cm.-hrs	cm.-hrs	cm.-hrs	cm.-hrs
CaCl <sub>2</sub> , gm. per liter	0.8	0.009	0.109	0.162	0.422	0.408
	0.6	0.013	0.283	0.523	0.398	0.126
	0.4	0.012	0.433	0.390	0.080	0.306
	0.2	0.010	0.549	0.259	0.293	0.012
	0.0	0.012	0.033	0.012	0.002	0.001

but that the tonic contraction is not always evident. Excess calcium has often been shown to produce tonic contraction in many types of muscle tissue. This same response seems to be at least partially demonstrated by the striated muscle of the insect foregut which was tested in this series of experiments.

When solutions lacked both Ca and K ions, muscle fibers of the crop very often rapidly lost their tonus and remained in a relaxed condition (Fig. 2, F).

#### *Effect of Changes in NaCl Concentration*

When NaCl concentrations were increased, higher osmotic pressure, with possible hypertonicity, became a factor to be considered. Several investigators have reported osmotic pressures of insect hemolymphs. Most of the work involved a freezing point technique with resulting values expressed as per cent NaCl necessary to produce the same freezing point depression and, consequently, the same osmotic pressure. Maluf (19), in 1939, summarized certain of the information concerning osmotic pressure in insect body fluids. He quoted values ranging between 0.8 per cent and 1.5 per cent NaCl, with a

mean of about 0.9 per cent. Backman (1) reported values between 0.8 per cent and 1.9 per cent NaCl for certain water beetles. For honey bee larvae, Bishop (3) stated that the osmotic pressure was equivalent to 1.5 per cent NaCl. Krogh (18) questioned the correctness of these high osmotic values obtained for insect hemolymph. No osmotic values for cockroach hemolymph have been recorded. However, in 1939, Yeager (24) used as high as 1.19 per cent NaCl in his work with the isolated dorsal vessel of the American roach, and Griffiths and Tauber (13) recommended 1.46 per cent NaCl as optimum for the excised foregut of the same insect. In view of these facts, it appears likely that osmotic pressure values above those equivalent to 1.0 per cent NaCl may very well be isotonic with certain insect hemolymphs.

It has been demonstrated with different muscle tissues that NaCl concentrations can be varied considerably with little or no effect upon activity of contracting tissue. Cole (8a) found that decreases and increases in osmotic pressure, up to 15 per cent, caused no significant changes in the rate and character of lobster heart beat, for several hours. Concerning the use of NaCl with the frog heart, BurrIDGE (4, p. 25) stated, "we find divers workers obtaining satisfactory percentages varying from 0.6 to 0.75 per cent." Clark (8), in 1913, varied NaCl from 0.5 per cent to 0.7 per cent with no significant effect upon the contracting frog heart. However, NaCl increases which produced definitely hypertonic solutions caused decreased muscular activity, according to Carlson (6, 7), Howell (17), Steggerda (22), and Cardot (5). It appears that slightly hypertonic solutions may produce little or no effect, but with increased hypertonicity, there is subsequent depression of activity.

To find how roach foregut muscles react to varying NaCl concentrations, the seven KCl/CaCl<sub>2</sub> ratios which gave the better activity products when combined with 1.0 per cent NaCl (in Table I) were further tested with 1.2 per cent, 1.3 per cent, 1.4 per cent, 1.5 per cent, 1.6 per cent, 1.7 per cent, and 1.8 per cent NaCl. All these solutions contained 0.02 per cent NaHCO<sub>3</sub>. The average activity product for these 56 mixtures is given in Table II. Each solution mean is an average for five crops from male and five crops from female roaches. In addition, the combined means for the eight NaCl levels, and similar means for the seven KCl/CaCl<sub>2</sub> ratios, are presented.

Information summarized in Table II raises the question as to what NaCl level represents an isotonic condition for the foregut of *Periplaneta americana*. Comparatively satisfactory results were obtained between 1.0 per cent and 1.8 per cent NaCl. This is an extremely wide range. However, activity products obtained with 1.4 per cent, 1.5 per cent, and 1.6 per cent NaCl were definitely superior to other sodium levels. This finding is in agreement with preliminary results (13). If high average activity products, derived from actual measurement of muscle responses, may be interpreted as indicative of isotonicity, solutions which contain 1.4 per cent to 1.6 per cent NaCl seem to approach ro-

tonicity for muscle in the foregut of the American roach. However, the apparent abnormal lowering of activity at the 1.5 per cent level complicates the problem. This average activity product dropped about 20 per cent below the average of the other two. In 1940, Griffiths and Tauber (13), with more tests per solution, found decreased activity in solutions which contained 1.62 per cent NaCl, as compared to those which contained 1.46 per cent. Thus, it seems possible that more extensive experimentation would show that the average activity product at the 1.6 per cent level of the present series of trials is too high. There is considerable evidence which points to this view. Four of the seven

TABLE II  
Average Activity Products for Solutions with Seven KCl/CaCl<sub>2</sub> Ratios and Eight NaCl Concentrations

		KCl/CaCl <sub>2</sub> concentrations, gm. per liter							Means
		$\frac{0.2}{0.4}$	$\frac{0.4}{0.6}$	$\frac{0.6}{0.8}$	$\frac{0.2}{0.2}$	$\frac{0.4}{0.4}$	$\frac{0.6}{0.6}$	$\frac{0.8}{0.8}$	
		Ratio of KCl/CaCl <sub>2</sub>							
		0.5	0.66	0.75	1.0	1.0	1.0	1.0	
NaCl, per cent	1.0	0 443	0 523	0 422	0 549	0 390	0 398	0 406	0 447
	1.2	0 552	0 296	0 767	0 382	0 475	0 689	0 377	0 505
	1.3	0 657	0 617	0 715	0 505	0 607	0 395	0 309	0 544
	1.4	1 169	0 755	0 637	1 286	0 835	0 550	0 330	0 795
	1.5	1 339	0 671	0 481	0 497	0 504	0 465	0 494	0 636
	1.6	1 546	1 109	0 240	0 686	0 600	0 642	0 459	0 755
	1.7	0 565	0 789	0 466	0 756	0 583	0 662	0 390	0 602
	1.8	0 524	0 910	0 260	0 477	0 407	0 678	0 343	0 514
Means		0 849	0 709	0 499	0 642	0 550	0 560	0 389	

KCl/CaCl<sub>2</sub> ratios tested with 1.4 per cent NaCl yielded average activity products greater than 0.750. In contrast, there was only one solution for the 1.5 per cent level, and only two for the 1.6 per cent level, which had activity products of this magnitude. In addition, and most important, the mixtures with 1.4 per cent NaCl produced the highest average activity product, namely, 0.795. Also, if the means at the right end of Table II are examined it will be seen that the deviation at the 1.5 per cent NaCl level is the only break in an activity curve from 1.0 per cent to 1.8 per cent NaCl. Therefore, it may be tentatively concluded that, of the NaCl concentrations tested, the one containing 1.40 gm per liter of solution offered the optimum environment for the isolated crop of *Periplaneta americana*. Additional evidence for this interpretation will be found in later paragraphs where other experimental data are presented.



Records from excised crops in 1.4 per cent NaCl are pictured in Fig. 2, A and B. Activity at this sodium level was usually characterized by a prompt initiation of contractions which were maintained over considerable periods of time. Some loss of tonus was usually manifested in the beginning. After small irregularities at first, the baseline is continued in a regular manner. Often, in mixtures in which ionic concentrations were apparently unbalanced, the amplitude, frequency, and baseline were extremely irregular, and activity time was of short duration. In test solution 33 (NaCl = 1.4 per cent, KCl = 0.02 per cent,  $\text{CaCl}_2$  = 0.04 per cent), where the activity time averaged more than 17 hours, one crop, No. 324, maintained contractions for almost 38 hours.

#### *Optimum KCl/ $\text{CaCl}_2$ Concentrations*

The KCl/ $\text{CaCl}_2$  ratio which allowed maximum activity was composed of 0.02 per cent KCl and 0.04 per cent  $\text{CaCl}_2$  (see Table II). Three of the five solutions, in which were obtained activity products of more than 1,000, contained KCl and  $\text{CaCl}_2$  in this ratio. The lowest average activity product recorded at this ratio was 0.443 for the solution which contained 1.0 per cent NaCl.

In general, Table II indicates that solutions with a KCl/ $\text{CaCl}_2$  ratio of less than 1.0 produced superior results when compared with solutions with a ratio of 1.0. It appears that, with a particular KCl/ $\text{CaCl}_2$  ratio, the activity of the excised foregut is decreased as the total amount of these two salts is increased. Thus, for the mixtures with a ratio of 1.0, the average activity product drops from 0.635 in the solutions containing 0.02 per cent KCl and 0.02 per cent  $\text{CaCl}_2$ , to 0.389 in those containing 0.08 per cent of each. In a similar manner, for the solutions with more calcium than potassium, the activity products shift from an average of 0.849 for mixtures with a KCl/ $\text{CaCl}_2$  ratio of 0.02 per cent/0.04 per cent to an average of 0.499 for the ratio of 0.06 per cent/0.08 per cent.

#### *Another Approach to Determine the Optimum NaCl Concentrations*

Since an optimum NaCl level could not be definitely determined by using data presented in Table II, additional experimentation with a different approach, and the use of a statistical analysis seemed expedient. In 1925, Glaser (11) found that the pH of American roach hemolymph lay within the range of 7.5–8.0. In the present experiment, test solutions containing 0.02 per cent  $\text{NaHCO}_3$  had a pH value which varied between 7.8 and 8.2. It was decided to test an additional series of NaCl levels at a pH of about 7.5. These mixtures were composed of four KCl/ $\text{CaCl}_2$  ratios (0.2/0.2, 0.4/0.4, 0.2/0.4, 0.4/0.6 gm per liter) and three NaCl levels (1.4, 1.5, and 1.6 gm per liter). To each solution was added 0.02 per cent  $\text{NaHCO}_3$ , and the mixture was then titrated with 0.1 normal HCl to a pH value of 7.5. Either while standing, or while flowing through the crop chamber, the pH of these solutions gradually rose to

between 7.8-8.0 within 10 to 14 hours. Thus, although crops were isolated at a pH of 7.5, and tests of activity began at this pH, muscular contractions continued as the OH ion concentration gradually increased. As before, five crops from each sex were tested in each mixture. Average activity products for each solution at pH 7.5 are given in Table III.

TABLE III  
Average Activity Products for Solutions at pH 7.5

NaCl	Per cent KCl/CaCl <sub>2</sub>				Means at pH 7.5
	$\frac{0.02}{0.02}$	$\frac{0.04}{0.04}$	$\frac{0.02}{0.04}$	$\frac{0.04}{0.06}$	
<i>per cent</i>	<i>cm - hrs</i>	<i>cm - hrs</i>	<i>cm - hrs</i>	<i>cm - hrs</i>	<i>cm - hrs</i>
1.4	0.656	0.980	0.725	0.462	0.705
1.5	0.503	0.285	0.751	0.555	0.524
1.6	0.606	0.575	0.175	0.534	0.473
Means at pH 7.5	0.588	0.613	0.550	0.517	0.568

TABLE IV  
Average Activity Products for Solutions at pH 8.0

NaCl	Per cent KCl/CaCl <sub>2</sub>				Means
	$\frac{0.02}{0.02}$	$\frac{0.04}{0.04}$	$\frac{0.02}{0.04}$	$\frac{0.04}{0.06}$	
<i>per cent</i>	<i>cm - hrs</i>	<i>cm - hrs</i>	<i>cm - hrs</i>	<i>cm - hrs</i>	<i>cm - hrs</i>
1.4	0.923	0.738	1.165	0.891	1.011
1.5	0.683	0.564	0.991	0.835	0.753
1.6	0.664	0.633	0.897	0.789	0.589
Means	0.790	0.645	1.351	0.845	0.784

As a basis for comparison, another series of five crops from each sex was tested in a set of mixtures with an initial pH of 8.0. These results are given in Table IV.

It is evident from Tables III and IV that there is a real difference between mixtures tested at pH 7.5 and pH 8.0 with the latter seemingly better. However, at both levels of alkalinity, optimum NaCl concentration for muscle contractions is 1.4 per cent and there is a reduction in crop activity with each increase of NaCl. These results are in accord with the previous suggestion that 1.4 per cent represents the optimum NaCl concentration. At pH 8.0, the KCl/CaCl<sub>2</sub> ratio with the highest average activity product was again the 0.02 per cent/0.04 per cent ratio.

To obtain a better understanding of the interactions which played a part in

the experiments, data from the four KCl/CaCl<sub>2</sub> ratios (0.2/0.2, 0.4/0.4, 0.2/0.4, and 0.4/0.6 gm per liter) tested in combination with 1.4 per cent, 1.5 per cent, and 1.6 per cent NaCl at pH values of 7.5 and 8.0 were statistically analyzed by the method of analysis of variance (20). The summary of this analysis is presented in Table V. Due to difficulties involved in interpretation, other subclass interactions than those listed were not tested. Subclasses in this analysis involved crops from a particular sex, tested on a given day, in any one solution. Test solutions had been used for 2 days of experimentation, and

TABLE V  
*Analysis of Variance for Solutions at pH 7.5 and pH 8.0*

Source of variation	Degrees of freedom	Sum of squares	Mean square
Total	239	125.66	
Within subclass (sampling error)	144	56.01	0.39
pH	1	3.81	3.81*
Sex	1	0.77	0.77
K/Ca	3	3.72	1.24*
NaCl	2	1.88	0.94†
Days	1	10.14	10.14*
pH and sex	1	3.34	3.34*
pH and K/Ca	3	7.93	2.64*
Sex and K/Ca	3	2.01	0.67*
pH and NaCl	2	4.11	2.06*
Sex and NaCl	2	2.91	1.46*
K/Ca and NaCl	6	3.38	0.56
Days and pH	1	3.55	3.55*
Sex and days	1	1.70	1.70†
K/Ca and days	3	1.32	0.44
NaCl and days	2	1.47	0.74
Discrepance (error)	63	17.61	0.28

\* Highly significant.

† Significant.

a preliminary survey of data had indicated a possible difference between crop activity on the 1st and 2nd day of the solution's use, in spite of the fact that the pH was adjusted at the beginning of each day's run.

Although the average activity product for crops from males (0.790) was greater than that from females (0.700), there was no significant difference. Crops tested on the 1st day yielded an activity product of 0.950 in contrast to 0.530 on the 2nd day. However, when the interaction between these two variables (sex, and day-tested) was analyzed, it was found that while the crops from males were considerably less active in day old solution (activity product 1.100 to 0.800), the females' crop activity was greater on the 2nd day (0.490 to 0.560). At present no adequate explanation can be offered for this response.

Since there is a significant difference between NaCl levels, and between KCl/CaCl<sub>2</sub> ratios, and since 1.4 per cent NaCl and 0.02 per cent KCl/0.04 per cent CaCl<sub>2</sub> produced the highest average activity products, it may be concluded that, under the conditions prevailing, these percentages form the medium which affords an optimum environment for activity of the excised crop of the American roach.

With these factors of NaCl concentration and KCl/CaCl<sub>2</sub> ratio rather well circumscribed, further experimentation upon the effect of pH appeared to be desirable. Therefore, four KCl/CaCl<sub>2</sub> ratios (0.2/0.2, 0.4/0.4, 0.2/0.4, and 0.4/0.6 gm. per liter) were tested with 1.4 per cent NaCl at initial pH values of 6.8 and 8.9 and the results were compared with the comparable solutions at pH 7.5 and 8.0.

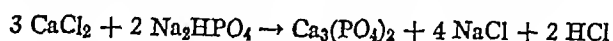
### *An Optimum pH Value*

In order to study the effect of changes in pH, solutions to be tested were made up with either phosphate or bicarbonate. Standard HCl or NaOH was added and solutions were titrated by means of a glass electrode to the desired pH. It was found that the pH of acid solutions (pH 6.0-6.5) which were buffered with both phosphate and bicarbonate, or bicarbonate alone, tended to rise rapidly to a pH above 7.0. Since this shift was undesirable, only phosphate was used in acid solutions, at the rate of 0.5 cc. of 0.25 normal NaH<sub>2</sub>PO<sub>4</sub> per liter. After titration, it was possible to maintain a pH between 6.5 and 6.9. Occasionally, however, the acidity increased and it was necessary to check the pH regularly and remedy any change.

Alkaline solutions were adjusted by adding 0.1 N NaOH to a mixture which contained 0.2 gm. of NaHCO<sub>3</sub> as a buffer. If phosphate was used, the pH dropped toward neutrality. If it was omitted, there was only a gradual lowering toward 8.0. Thus, it was possible to maintain a pH above 8.5 for some 12 to 15 hours without a change of solution. The pH values were adjusted at the beginning of each day's run, and at any other time when necessary.

The peculiar shifts in pH noted above are apparently related to the CO<sub>2</sub> exchange between the atmosphere and the test solutions. Although fluctuations occurred both while standing in glass containers and during the course of experimentations, the change was more rapid when the solutions were being aerated and were flowing through the crop chamber. Changes were essentially the same whether insect tissues were present or absent in the experimental set up. Apparently, agitation by bubbling and movement of the fluid afforded a better opportunity for the loss or uptake of the CO<sub>2</sub>, depending on the pH of the medium. In acid solutions, part of the NaHCO<sub>3</sub> was changed to unstable H<sub>2</sub>CO<sub>3</sub>. It appeared that this compound decomposed to form water and CO<sub>2</sub>. The latter was lost, with the resulting rise in the pH. However, in a medium with a pH above 8.0 there was no loss, but,

CO<sub>2</sub> Uptake of CO<sub>2</sub> was slow and the resultant pH change was, therefore, gradual. The greater rapidity of change in the presence of phosphates may be partially explained. The buffer effect of phosphates is at a minimum at a pH value of about 8.5. This is due to the fact that the tri-sodium form is changing to the di-sodium form at this value. In addition, if CaCl<sub>2</sub> is present, the following reaction will take place when the mixture is alkaline



However, since the quantities of both reactants were small, the amount of HCl would be very limited. Other factors may be contributing to the shift of pH, but no further explanation can be offered at this time.

Various workers (4, 5, 7, 8, 9, 17) have demonstrated that changes in acidity or alkalinity affect the activity of muscle tissues. These investigators have used different kinds of muscle from several widely separated animal groups. With experiments performed upon different animals and with techniques not the same, results may often be seemingly contradictory. Nevertheless, certain tendencies have made themselves evident.

A review of the literature (12) indicates that moderate and sometimes rather large changes in pH do not materially affect the normal activity of contracting muscle. However, within certain limits, there is a tendency for increase in acidity to depress, and increase in alkalinity, also within a definite range, to stimulate activity. It is probable that the stimulatory effect is a temporary one and that this concentration of hydroxyl ions would prove detrimental if maintained over longer periods, or if the OH<sup>-</sup> ions were further increased. These same tendencies were noted in the present experiments with roach fore-gut muscle.

Results from the crops tested in 1.4 per cent NaCl at pH values of 6.8 and 8.9 are compared in Table VI with those at 7.5 and 8.0. It should be remembered that crops in solutions which were initially tested at pH 7.5 and 8.9 continued their activity even though the solutions gradually approached pH 8.0. This change must have affected the activity in these solutions and should be considered when studying data in Table V.

Data of Table VI were statistically analyzed. The summary of this procedure is in Table VII.

Several significant differences may be noticed in Table VII. There was a significant sex difference, and the activity for crops from males (0.850) was better than that for crops from females (0.640). Once again the difference between day's run was highly significant. The interaction between sex and day's run was highly significant and, as noted previously, the activity for males was markedly reduced on the 2nd day (1.160 to 0.540) while the females changed very little (0.630 to 0.640).

Until further evidence may be produced, it has been assumed that the change

in concentration of the phosphate and bicarbonate anions was not responsible for the different results found at the four pH levels, but rather that the differences were caused by a shift in the H ion concentration. Average activity products produced at the four pH values differ significantly. That at a pH of 8.0 is greatest. Therefore, it may be concluded that this value represents

TABLE VI

*A Comparison of Records from Solutions with pH Values of 8.9, 8.0, 7.5, and 6.8*

pH	Solution No.	KCl/CaCl <sub>2</sub>	Amplitude	Activity	Activity product
		<i>gm. per liter</i>	<i>cm.</i>	<i>hrs.</i>	<i>cm.-hrs.</i>
8.9	79	0.2/0.2	0.030	10.3	0.324
	80	0.4/0.4	0.066	12.1	0.896
	81	0.2/0.4	0.032	19.7	0.691
	82	0.4/0.6	0.060	18.9	1.075
	Mean		0.047	15.3	0.797
8.0	33	0.2/0.2	0.071	17.4	1.286
	34	0.4/0.4	0.067	11.5	0.835
	37	0.2/0.4	0.066	15.2	1.169
	38	0.4/0.6	0.059	9.8	0.755
	Mean		0.066	13.5	1.011
7.5	41	0.2/0.2	0.042	18.1	0.656
	42	0.4/0.4	0.058	15.4	0.920
	45	0.2/0.4	0.044	12.7	0.725
	46	0.4/0.6	0.027	9.8	0.462
	Mean		0.043	14.0	0.706
6.8	75	0.2/0.2	0.077	10.8	0.879
	76	0.4/0.4	0.066	9.2	0.669
	77	0.2/0.4	0.028	7.1	0.245
	78	0.4/0.6	0.053	6.3	0.351
	Mean		0.056	8.4	0.536

the optimum one, of those tested, for the activity of the excised crop of the American roach.

Crop 814, in solution 82, with an initial pH of 8.9, produced an unusual record. At the end of 24 hours of activity, its amplitude had dropped almost to zero. At that time the pH was 8.0. The solution was changed, the pH was again 8.9. Instead of showing an inhibitory effect, contractions increased in magnitude and the best part of the record was made during the next 15 hours.

Altogether, activity had continued over a period of almost 47 hours. No adequate explanation can be offered, but, apparently, the muscle had undergone some change whereby the increased alkalinity served as a stimulus for greater activity. This phenomenon may be allied with the stimulatory effect of the hydroxyl ion which was noted by Carlson (6, 7) and de Burgh Daly (9). A similar but less spectacular result was obtained from crop 820 in the same kind of solution.

TABLE VII  
*Statistical Analysis for Solutions Tested at pH Values of 8.9, 8.0, 7.5, and 6.8*

Source of variation	Degrees of freedom	Sum of squares	Mean square
Total	159	81.34	
Within subclass (sampling error)	96	40.38	0.43
Sex	1	2.00	2.00*
pH	3	4.52	1.51*
K/Ca	3	0.78	0.26
Days	1	3.69	3.69†
pH and sex	3	2.12	0.71
Sex and days	1	3.83	3.83†
pH and K/Ca	9	7.46	0.83
pH and days	3	1.31	0.44
Days and K/Ca	3	2.43	0.81
Discrepancy (error)	33	11.52	0.35

\* Significant.

† Highly significant.

### *The Sex Difference*

In their preliminary study of the motility of the isolated roach foregut, Griffiths and Tauber (13) stated, "The crops from male roaches produced significantly higher activity products than those from females. Food was present in the foregut of the females more often than in that of the males." In addition, they observed that the quantity of food contained in the crops did not seem to be correlated with the activity of an individual foregut, that is, a large amount of food did not depress activity. It was suggested that reduced activity of crops from female roaches was responsible for reduced motility. The same correlations were noted in the present investigation.

For solutions with pH values of about 8.0, average activity products of crops from males were greater than those from females in 52 of the 74 mixtures. Thus, in slightly more than 70 per cent of the cases, crops from males produced the higher average activity products. If, for the same 74 solutions, the average of all of the activity products for males and females is determined, it is found that the foreguts from males averaged 0.525 and those from females averaged

0.399 The product for males was almost 25 per cent greater than that for females. In the two sets of data which were treated statistically, the average activity product for crops from males was greater than that for crops from females and in one instance the difference was significant. It becomes obvious, therefore, that there is some fundamental difference between the two sexes as regards the motility of the excised foregut.

During the course of the present experimentation, certain modifications in technique were made in the hope that some adequate interpretation of this difference could be advanced. To determine whether there was a significant sex difference in the quantity of food ingested, groups of five males and five

TABLE VIII  
*Average Amounts of Food Contained in the Crops of 445 Roaches*

Time since fed <i>hrs</i>	Male		Female	
	No. of tests	Average amount food	No. of tests	Average amount food
16	12	1.92	15	2.07
17	30	1.33	24	2.25
18	35	0.94	44	2.18
19	27	1.37	35	2.00
20	32	0.91	28	2.21
21	22	0.64	33	1.76
22	24	1.13	29	2.38
23	20	0.70	11	1.36
24	20	0.30	4	0.25

\* 4.00 represents a full crop and 0.00 represents an empty one.

females were isolated for 3 days and were then allowed access to food for 1 hour. Banana paste, whole wheat bread which had been soaked in water, and moistened starch were used as test foods. 50 males and 50 females in groups of five were fed on each diet. There was no significant difference in the amount of food ingested by the two sexes.

Most roaches used in the gut motility experiments were isolated for 48 hours and were then allowed access to moistened starch for several hours. The starch had been mixed with carmine so that the ingested food could be identified in the gut. Roaches were usually used for experimentation on the day following this feeding. When the foregut was removed, the amount of food in the crop was recorded. If food was present in only one-fourth of the crop it was recorded as 1, if half of the crop was full, it was listed as 2, if three-fourths full, as 3, and if completely full, as 4. Data for 445 roach crops are presented in Table VIII. This includes information concerning the number of roaches and



the amount of food which remained in the crops from 16 to 24 hours after feeding had ceased. In only one instance (at 24 hours) was the average amount of food contained by the crops greater for males than for females.

Snipes and Tauber (21) reported that for *Periplaneta americana* the average egestion time was 19.6 hours for males and 21.4 hours for females. While this was not a significant difference, the egestion time discrepancy may have been related to the motility difference observed in excised crops from the American roach. Table VIII clearly shows a significant difference in the rate of progression of food in crops from males and females, and, consequently in the amount of food remaining in the foregut. This food progression rate is more rapid in the foreguts from the male roaches.

As stated above, there is a significant sex difference in activity products of crops from males and females, in the amount of food contained in the crops, in the rate of progression of food through the ingluvial region, and in the reaction to the age of the salt solution. However, the amount of food ingested by males and females is not significantly different. Therefore, it appears that variations in motility are produced by inherent activity differences which are normally present in the ingluvial region of the American roach. That the two sexes of the American roach differ in other features has been reported previously by Griffiths and Tauber when they found differences in the duration of the nymphal period (14), the number of molts (14), and the adult longevity (15).

#### SUMMARY AND CONCLUSIONS

1 Twenty-five solutions which contained KCl (0.0, 0.2, 0.4, 0.6, and 0.8 gm per liter), in combination with  $\text{CaCl}_2$  (0.0, 0.2, 0.4, 0.6, and 0.8 gm per liter), 10.0 gm of NaCl, and 0.2 gm of  $\text{NaHCO}_3$  per liter of solution were tested in order to determine satisfactory KCl/ $\text{CaCl}_2$  ratios in an insect physiological salt mixture for the maintenance of muscular activity by the isolated crop of the American roach.

Satisfactory activity products (0.390 to 0.549) were obtained in seven mixtures with KCl/ $\text{CaCl}_2$  ratios of 0.2/0.2, 0.4/0.4, 0.6/0.6, 0.8/0.8, 0.2/0.4, 0.4/0.6, and 0.6/0.8, expressed as gram per liter. These ratios lie between 0.50 and 1.00.

In solutions which contained calcium, but no potassium, approximately 50 per cent of the crops exhibited an initial tone increase and were arrested in rigor. See Fig. 2.

In solutions which contained potassium, but no calcium, all crops showed an initial loss of tone and arrest in relaxation. See Fig. 2.

2 Seven KCl/ $\text{CaCl}_2$  ratios (see paragraph 1 above) were tested with eight NaCl concentrations (1.0, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, and 1.8 per cent) at a pH of 8.0. In these mixtures, the ones with KCl/ $\text{CaCl}_2$  ratios of less than 1.0 produced higher activity products than those with ratios equal to 1.00. The

highest average activity product (0.849) was obtained in the solutions with 0.2 gm of KCl and 0.4 gm of  $\text{CaCl}_2$  per liter

3 Four KCl/ $\text{CaCl}_2$  ratios (0.2/0.2, 0.4/0.4, 0.2/0.4, and 0.4/0.6 gm per liter) were tested with 1.4, 1.5, and 1.6 per cent NaCl at a pH of 7.5. When analyzed with data from comparable solutions at a pH of 8.0, it was found that 1.4 per cent NaCl afforded an optimum environment for isolated crop activity.

4 Effects of hydrogen and hydroxyl ion concentrations were studied at pH values of 6.8, 7.5, 8.0, and 8.9. The highest average activity product, 1.011, was produced at a pH of about 8.0.

5 A satisfactory physiological salt solution for the isolated foregut of the American roach, *Periplaneta americana*, would contain 14.0 gm. of NaCl, 0.4 gm. of  $\text{CaCl}_2$ , 0.2 gm. of KCl, and 0.2 gm. of  $\text{NaHCO}_3$  per liter of solution. This mixture should have a pH value between 7.8 and 8.2.

6 Durations of crop activity extending over periods as long as 25 hours were quite common, and several crops maintained contractions for more than 30 hours. The greatest longevity was for crop 814, from a female, which continued activity for slightly more than 47 hours.

7 A significant difference between the activity products of the crops from males and the crops from females was recorded. Although there was not a significant difference in the amount of food ingested by males and females, 12 hours after feeding there was more food in the females' crops, and the food progressed more rapidly through the males' crops than through the females'. In addition, crops from the two sexes reacted differently to the effects of day old solutions. This sex difference is apparently related to an inherently increased activity of the crop from the male roach.

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## ZONE BEHAVIOR OF ENZYMES

ILLUSTRATED BY THE EFFECT OF DISSOCIATION CONSTANT AND DILUTION  
ON THE SYSTEM CHOLINESTERASE PHYSOSTIGMINE\*

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### INTRODUCTION

The study of systems composed of an enzyme and reversible inhibitor has contributed greatly to our knowledge of enzyme kinetics. Such systems are of importance in the field of pharmacology, since a number of drugs act by inhibiting known enzyme systems, while many of those whose biochemical mode of action is still unknown may operate in similar fashion.

The classical treatment of the kinetics of enzyme reactions has been based upon the assumption of a very small concentration of enzyme centers acting according to the laws governing first order reactions (pseudomonomolecular). In this paper we shall show that under a number of common conditions such treatment cannot adequately describe the behavior of the system but that a more complete analysis must be employed. Enzyme inhibitor and enzyme-substrate systems will be shown to behave in three distinct ways depending upon the concentrations of the reactants and the dissociation constant of the system. The boundaries of these three 'zones of behavior' will be established on a kinetic basis applicable to all such systems, and the qualitative and quantitative differences in behavior will be demonstrated.

An important practical consequence of the theory of zone behavior concerns the effect of diluting a mixture of enzyme and inhibitor (or substrate). It is common practice to remove serum from an animal which has received some drug, and then, after appropriate dilution, to determine *in vitro* the degree of inhibition produced in some serum enzyme. It is then assumed that the observed degree of inhibition obtained in this manner is representative of the state of the enzyme in the animal's circulating serum before removal. It will be shown, however, that dilution is a crucial operation which significantly affects the subsequent experimental observations, and that a conversion equation (or conversion curves) must be used if the usual experimental data are to be applied to the situation existing *in vivo*.

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The general methods presented in the first part of this paper will then be applied in an illustrative fashion to the system cholinesterase-physostigmine, which will be shown both qualitatively and quantitatively to exhibit the predicted behavior on dilution

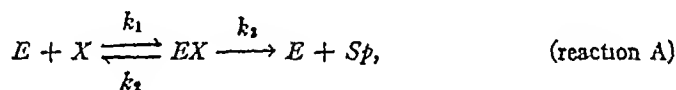
In the final section we shall discuss (a) the evolution of the concept of zone behavior, (b) further conclusions of biological importance, (c) an alternative definition of the zone boundaries, (d) extension of the analysis to more complex systems, and (e) certain significant limitations on the analysis here presented

## THEORETICAL

### *Derivation of the General Equation*

In studying any enzyme from a kinetic standpoint, the observed data are necessarily based upon (a) the concentration of a substance,  $X$ , that combines with the enzyme,  $E$ , to form a complex,  $EX$ , and (b) the rate of reaction at which breakdown products,  $Sp$ , are formed from  $EX$  with the liberation of  $E$ . The substance,  $X$ , may be either a substrate or an inhibitor, depending on the behavior of the complex,  $EX$ , and it is necessary to define precisely what is meant by these terms

Let us consider the combination of enzyme  $E$  with a single molecule of  $X$  to form a complex  $EX$



where  $k_1$ ,  $k_2$ , and  $k_3$  are velocity constants, and  $Sp$  represents the split products of  $EX$  breakdown. In this reaction we call  $X$  a substrate if the complex  $EX$  breaks down to form  $Sp$  at a rate that is *not* negligible for the purposes under consideration. If, on the other hand, the breakdown of  $EX$  is negligible ( $k_3 \ll k_2$ ), we call  $X$  a reversible inhibitor.

It is evident from this that the difference between a substrate and a reversible inhibitor is determined only by the relation of the velocity constants in reaction A. It also follows that all reversible inhibitors whose action is upon the same enzyme centers as normally would combine with substrate molecules are necessarily *competitive* inhibitors. The *degree* of competition will naturally vary, but whether it be considerable (as when one substrate "inhibits" the breakdown of another) or very slight (as when a potent drug combines with a substantial number of enzyme centers), there is no basic difference in the kinetic mode of action. For purposes of simplicity, however, this paper will limit itself to the case where competition is negligible or truly absent (see Discussion, p. 583).

Let  $E$ ,  $I$ ,  $S$ ,  $EI$ , and  $ES$  now represent the total molar concentrations of enzyme centers, inhibitor, substrate, and their complexes respectively. Then,

if  $v$  is the observed velocity of substrate breakdown, it is true under all circumstances that

$$v = k_1(ES)$$

The concentration of complex,  $ES$ , cannot be measured directly. If, however, a large excess of substrate is added, in accordance with the principle of mass action, virtually all the enzyme will combine with it to form the complex,  $ES$ , so that  $ES \approx E$ , and the enzyme remaining free,  $E - ES \approx 0$ . Under these circumstances, further increase in  $S$  can produce no increase in  $ES$ , and so no increase in  $v$ , then

$$v_{\max.} = k_1 E$$

If an inhibitor be present, a fraction of the total enzyme will combine with it to form the inactive complex,  $EI$ , and the amount of enzyme left free to combine with an excess of substrate will be  $(E - EI) = ES$ . Substituting this value of  $ES$ ,

$$v = k_1(E - EI)$$

and then dividing one equation by the other,

$$\frac{v}{v_{\max.}} = \frac{k_1(E - EI)}{k_1 E} = 1 - \frac{EI}{E}$$

If we now let  $i$  represent the fraction of the total enzyme that is combined with inhibitor,

$$i = \frac{EI}{E}$$

then from the above,

$$i = 1 - \frac{v}{v_{\max.}} \quad (1)$$

The fractional inhibition,  $i$ , of an enzyme can therefore readily be found, since both  $v$  and  $v_{\max.}$  are measurable quantities,  $i$  will vary between the limits 0 and 1 as  $v$  varies from  $v_{\max.}$  to 0.

The reaction between enzyme and inhibitor (reaction A) becomes entirely equivalent to



since breakdown of the combined form is negligible. If the law of mass action is followed, then at equilibrium,

$$\frac{(E - EI)(I - EI)}{(EI)} = \frac{k_2}{k_1} = K$$

where  $K$  is the dissociation constant of the complex. Substituting the value  $EI = iE$  (since  $i \equiv EI/E$ ), and simplifying,

$$I = \frac{K_2}{1 - i} + iE \quad (2B)$$

This equation states that the *total* molar concentration of inhibitor ( $I$ ) is equal to the sum of two parts. One of these,  $iE$ , will be recognized as equivalent to  $EI$ , the molar concentration of *combined* inhibitor. It follows that the other part,  $\frac{K_2}{1 - i}$ , must represent the molar concentration of *free* inhibitor. Equation 2B then says simply that total inhibitor equals free plus combined forms.

Now it will be obvious that if the enzyme concentration<sup>1</sup> is very small practically all the inhibitor is present in the free form. On the other hand, if enzyme concentration is very great, nearly all the inhibitor will be in the combined form (except at extreme values of  $i$ ). It should thus be possible to introduce working simplifications of the equations by neglecting combined inhibitor, on the one hand,

$$I = \frac{K_2}{1 - i} \quad (2A)$$

or free inhibitor, on the other,

$$I = iE \quad (2C)$$

for each of the two cases considered. However, it is clear from inspection of the equation that these simplifications cannot really be justified on the basis of the actual magnitude of the enzyme concentration  $E$ , but rather by its magnitude *relative to*  $K$ .

The dissociation constant  $K$  here has the dimensions of concentration and is usually expressed in molar units. It is a constant for any given enzyme-substrate or enzyme-inhibitor system, provided only that all the physical conditions not mentioned in the equation, such as temperature, pH, choice of reactants, and so on, are held constant. Conversely,  $K$  may vary continuously if temperature or pH changes, or discontinuously if one enzyme, substrate, or inhibitor is substituted for another.

The use of simplified forms of the equation describing the kinetic behavior of *all* enzyme-inhibitor systems of the general type under consideration has just been shown to depend upon the ratio  $E/K$ , and not upon absolute concentrations of enzyme or inhibitor. If we therefore express  $E$  and  $I$ , not in molar concentrations, but *using*  $K$  as our unit for whatever system we deal

<sup>1</sup>  $E$  is the total molar concentration of enzyme centers, irrespective of the number of centers that may be carried by a single protein molecule.

with, we will thereby generalize our discussion to apply to any enzyme-inhibitor system.<sup>2</sup>

The term  $E/K$  we shall call the "specific concentration" of enzyme and designate as  $E'$ . Similarly,  $I/K$  is the "specific concentration" of inhibitor, designated by  $I'$ .

Dividing equation 2 B by  $K$  and substituting, we now have

$$I = \frac{i}{1-i} + iE' \quad (3B)$$

For the case where specific concentration of enzyme ( $E'$ ) is small, and practically all the inhibitor is free, this becomes

$$I' = \frac{i}{1-i} \quad (3A)$$

It is evident that in this case the inhibition is a function of specific concentration of inhibitor alone, and independent of enzyme concentration.

For the case where  $E'$  is large and practically all the inhibitor is combined, the equation becomes

$$I' = iE' \quad (3C)$$

Here the inhibition is a function of specific concentration of inhibitor and enzyme, being equal to the ratio  $I'/E'$ .

We now see that equation 3 in its three forms describes the behavior of all enzyme-inhibitor systems acting according to reaction B. Furthermore, since nothing has been added which is not implicit in the mass action law, it is equally valid for any system—chemical, physical, or biological—where two reactants combine reversibly in a manner described by this law.

### *Zones of Enzyme Behavior*

The three forms of equation 3 represent three distinct zones of enzyme behavior, hereafter designated A, B, and C, after the equations which define them. It will be necessary now to determine more exactly the boundaries of these three zones of behavior. How "large" or how "small" must  $E'$  be in order that equation 3A or 3C instead of the full form 3B may be used to describe behavior adequately? The answer will depend upon how large an error  $\epsilon$  (or experimentally measured term) we are willing to accept. If we decided upon the maximum acceptable error ( $\Delta i$ ), we can then determine where the zone boundaries must lie in order that this error shall never be exceeded. It can be

<sup>2</sup> It will be recalled that  $i$  is  $EI/P$  a dimensionless number and hence is unaffected by changing the system of units employed.

<sup>3</sup>  $E'$  and  $I'$ , being ratios of concentration, are dimensionless numbers. The term "specific concentration" is arrived at by analogy to specific gravity (the ratio of density relative to that of water, taken arbitrarily as unity).



be evident that the more rigid we are in fixing  $\Delta i$ , the larger will be the zone in which the full equation 3 B must be used

Fig 1 shows the zone boundaries for three arbitrary values of  $\Delta i$ . Strictly interpreted, zone B lies between the pairs of boundary curves for any given  $\Delta i$ , and the zone boundaries are seen to vary with the fractional inhibition  $i$ . For working purposes, it is necessary to eliminate this variation with  $i$  and decide

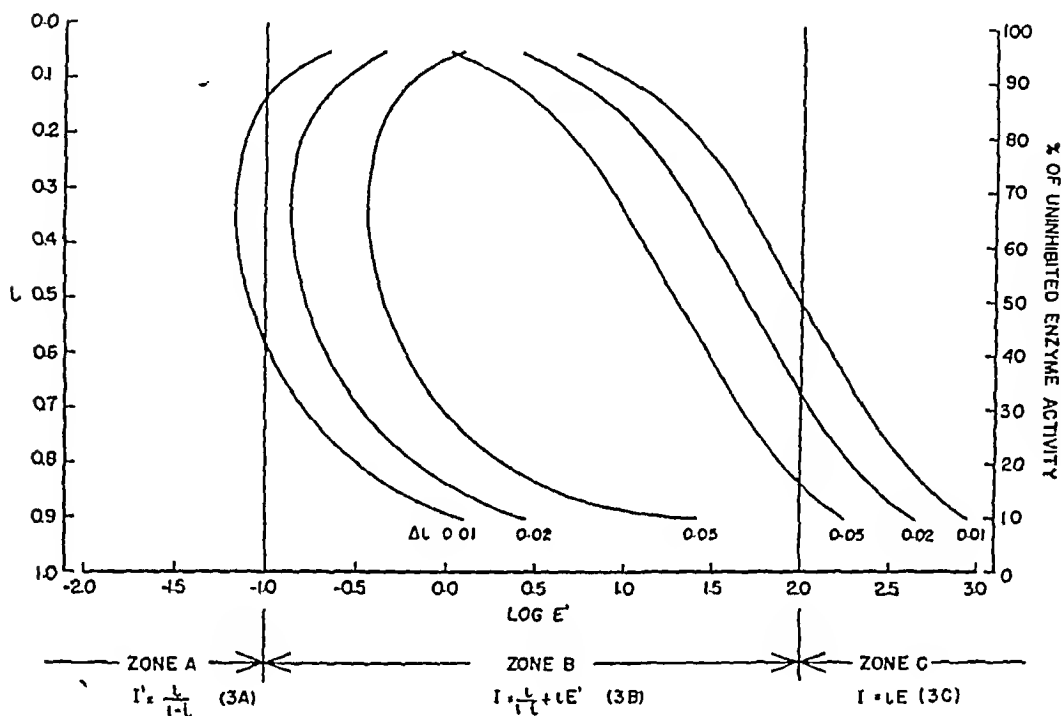


FIG 1 Zone boundaries Ordinate, fractional inhibition,  $i$  Abscissa,  $\log_{10}$  of the specific enzyme concentration ( $E'$ ) Each pair of curves shows the exact boundary of zone B for a given value of  $\Delta i$ . To the left of the region enclosed by each pair lies zone A, to the right, zone C. Straight vertical lines are approximate zone boundaries, when  $\Delta i = 0.01$ , neglecting the effect of variation of  $i$  on the boundary value of  $E'$ .

upon two boundary values of  $E'$  which will give the best approximation. In the case illustrated,  $\Delta i = 0.01$  was selected, and approximate boundary values are shown to be  $E' = 0.1$  to the left and  $E' = 100$  to the right.

The mathematical derivation of the boundary curves plotted on Fig 1 is as follows. At the boundary AB,  $I'$  in equation 3A plus the error caused by the increment  $\Delta i$  must equal  $I'$  in equation 3B.

Thus,

$$\frac{i + \Delta i}{1 - (i + \Delta i)} = \frac{i}{1 - i} + iE'$$

or

$$E' = \frac{\Delta i}{i} \frac{1}{(1 - \Delta i - i)(1 - i)} \quad \text{at boundary } AB$$

At boundary *BC*, *I'* in equation 3 *C* plus the error caused by the increment  $\Delta i$  must equal *I'* in equation 3 *B*, so that

$$(i + \Delta i)E' = \frac{i}{1 - i} + iE'$$

and

$$E' = \frac{i}{\Delta i} \frac{1}{1 - i} \quad \text{at boundary } BC$$

*Most Enzyme Systems Operate in Zone A* — Most of the general treatments of enzyme kinetics have hitherto been based on the assumption that the concentration of enzyme centers is constant and so small compared with the concentration of any substance with which it may combine that it may be neglected. This is the situation to which equation 3 *A* has been shown to apply. Michaelis and Menten (1), Haldane (2), Lineweaver and Burk (3), and others have all based their algebraic and graphic treatment upon this assumption and consequently have limited their discussions to zone A. Likewise the familiar Michaelis law applies only within this zone.<sup>4</sup>

Their failure to extend their fertile methods to zones B and C is due to the fact that most enzymes are studied in very dilute solution. There are several reasons for this. First, enzymes are considered to be protein molecules carrying only one or very few active centers per molecule, so that the factor of solubility precludes high molar concentrations of enzyme centers. Second, even if the enzyme can be concentrated to some degree, it is seldom technically convenient to measure the very high reaction velocities that occur in concentrated solution under optimal conditions. A survey of any list of dissociation constants such as that given by Haldane (2),<sup>5</sup> will show that in the great majority of instances *K* is greater than  $10^{-4}$  molar, with *E*, the concentration of enzyme, limited by the considerations just mentioned, *E*/*K* or *E'* will be less than 0.1, so that the systems lie in zone A.

#### *Systems in Zones B and C* —

1. When *K* is small. Since *E'*, which determines the zone of an enzyme system, is defined as *E*/*K*, it is clear that if *K* is small enough *E'* may be between 0.1 and 100, so that the system is in zone B, or may be greater than 100,

<sup>4</sup> The Michaelis law states that the concentration of inhibitor required for half inhibition is equal to the dissociation constant *K*; that is to say,  $I = K$  or  $I/K = 1$ , and  $I' = 1$  when  $i = 0.5$ . This is true only in equation 3 *A*.

<sup>5</sup> Haldane, J. B. S. *Enzymes*, London: Longmans, Green & Co. 1930, 35.

so that it is in zone C. A few enzyme-substrate complexes, such as peroxidases and "oxygenases," have dissociation constants of the order of  $10^{-6}$  to  $10^{-7}$ , so that  $E'$  might be greater than 0.1. Furthermore, a significant number of enzyme-inhibitor systems, exemplified by cholinesterase and physostigmine, have dissociation constants as small as or smaller than this. These may be expected to show zone B or C behavior *in vitro*. For a single enzyme studied at a single concentration,  $K$  will in general be different for the various substances that form complexes with it, and  $E'$  will vary inversely as  $K$ . Then for any two substances whose dissociation constants differ with respect to a single enzyme, it is possible that  $E'$  may in one case be less than 0.1, and in the other case greater than 0.1. The system will then be in zone A with respect to the first substance and in zone B or C with respect to the second.

2. When  $E$  is large. There is at least one situation where an enzyme may exist in relatively high concentration and yet not yield a reaction velocity that is technically unmanageable. Let us consider tissue slices or intact cells such as can be handled in the Warburg apparatus. These will be supposed to give conveniently measurable reaction velocities and to have been so handled that the enzyme distribution in the tissue has not been disturbed. There has accumulated much evidence that some enzymes, such as cholinesterase, are confined to a small fraction of the total number of cells, or are even confined to localized regions of a single cell. At these points of localization the molar concentration of enzyme centers may be very much higher than that indicated by a consideration of the total tissue or fluid volume involved. So long as the rate of the reaction measured is not limited by diffusion, the kinetic behavior might indicate that the system lay in zone B or C, even though the same total amount of enzyme would lie in zone A if it were dissolved throughout the total volume of the reaction mixture. If the differences of behavior exhibited by enzymes in the three zones could be experimentally detected, a means would be provided for estimating directly the enzyme concentration in the intact cell. Such differences of behavior will be pointed out below, together with certain practical tests for estimating specific enzyme concentrations.

### *Graphical Representation of the General Equation (3 B)*

*Description of Plot*—The usual representation of the action of a drug upon its receptor *in vivo* or *in vitro* is the plot of effect as ordinate against the logarithm of the concentration of the drug as abscissa. Equation 3 B is plotted in this way in Fig. 2, which shows the relation between the fractional inhibition  $i$  and the logarithm of the specific concentration of inhibitor  $I'$ . Each curve represents this function at a single value of the specific concentration of enzyme  $E'$ , these values being chosen arbitrarily for convenient spacing of curves. It will be observed that the curves representing successively lower specific concentrations of enzyme are asymptotic to a limiting curve that is

nearly reached when  $E'$  falls to 0.1. In the direction of increasing enzyme concentration the curves become steeper and parallel, the points of inflection occurring progressively nearer to the region where  $i = 1.0$ .

This figure provides a graphic example of the variations in behavior in each of the three zones, as previously discussed in connection with equation 3.

Zone A is represented by the limiting curve  $E' \leq 0.1$ , this curve representing all values of  $E'$  more dilute than 0.1. It follows from this curve that fractional inhibition depends only upon  $I'$  and is independent of  $E'$  within this zone. This plot also shows that  $I = K$  ( $I/K = 1 = I'$ ,  $\log I' = 0$ ) when  $i = 0.5$ , as postulated by Michaelis, only in this zone.

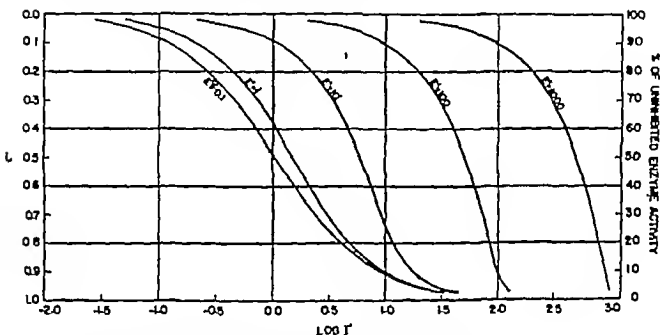


FIG. 2. Fractional inhibition,  $i$ , as a function of the  $\log_{10}$  of the specific concentration of inhibitor  $I'$  at various specific enzyme concentrations,  $E'$ .

Zone C is represented by the region to the right of the curve  $E' = 100$ . In this zone, the curves not only become parallel, but assume the shape of a simple logarithmic function. Furthermore, any two curves are separated by a distance which, measured off on the  $I'$  axis, is equal to the factor by which  $E'$  is changed between the two curves (e.g. the horizontal distance from the curve  $E' = 100$  to  $E' = 1000$  is just 1 log unit on the  $I'$  axis). As a consequence, the fractional inhibition ( $i$ ) for a given  $E'$  is directly proportional to the  $I'$  employed, and the inhibition ( $i$ ) is equal to the ratio of inhibitor to enzyme ( $I'/E'$  or  $I/E$ ) in the solution.

Zone B is, of course, represented by the area between the curves  $E' \leq 0.1$  and  $E' = 100$ , and here inhibition is a function of both  $I'$  and  $E'$  as stated by equation 3B.

Slope  $di/d \log I'$ —The slopes of the curves of Fig. 2 are of interest because they provide a useful criterion for determining whether a system follows mass

law requirements, and also for roughly estimating the specific enzyme concentration

If we consider the slope at  $i = 0.5$ , it will be evident from the form of the curves that this will be a minimum when  $E' \leq 0.1$ , and a maximum when  $E' \geq 100$  (since the curves have attained their maximum steepness at this latter point). These limiting slopes are actually found to be 0.575 and 1.151.<sup>6</sup> Since these curves apply very generally to all systems of the type represented by reaction B, it follows that any such system in whatever zone must yield slopes within these limiting slopes at  $i = 0.5$ . Conversely, failure to fall within these limits is a result of only two possibilities: systematic or random experimental error is present, or the reaction does not follow this type of equilibrium equation.

If the slope falls within these limits, assuming that the reaction does follow this type of equilibrium equation, substitution of the experimentally determined slope in equation 4 (setting  $i = 0.5$ ) will yield a preliminary value for  $E'$  and hence an indication of the zone in which the system lies. Because a small change in slope corresponds to a large change in  $E'$ , the slope is of more use as an exclusion test than for precise evaluation of  $E'$ .

The reader may have noticed the agreement between the limiting slope 0.575 in zone A and Van Slyke's (4) maximum molar buffering capacity,  $\beta_M$ , of any monovalent buffer. This is not a coincidence but rather a reflection of the fact that, like equations 2 and 3, the Henderson-Hasselbalch equation is derived directly from the mass action law.<sup>7</sup>

### Effect of Dilution

We will now consider the effect of diluting an enzyme solution (e.g., serum) containing a reversible inhibitor. It has long been realized that dilution of a

<sup>6</sup> The numerical values for slope are obtained by differentiating equation 3B with respect to  $\log I'$  and evaluating the limits when  $E' = 0$  and  $E' = \infty$ .  
Thus

$$\frac{di}{d \log I'} = 2.303i \left[ 1 - \frac{i}{1 + (1-i)^2 E'} \right] \quad (4)$$

<sup>7</sup> Written in arithmetic form, the Henderson-Hasselbalch equation becomes  $H^+ = \frac{K(1-\alpha)}{\alpha}$ . Since  $\alpha$  (defined as the ratio of free to total electrolyte) is equal to  $1-i$ , we may write  $H^+ = K_1/(1-i)$ , that is to say, free hydrogen ion equals  $K_1/(1-i)$ . This will be recognized as entirely analogous to the statement *free inhibitor equals  $K_1/(1-i)$*  (p. 562). These equalities are true for all zones, but since the curves of Fig. 2 are plotted against *total I*, they will depict the above functions *only where total I is equivalent to free I*, namely, in the limiting zone A curve, where  $E' \leq 0.1$ . This single curve, then, represents the Henderson-Hasselbalch equation, and it is quite natural that its slope, 0.575, at  $i = 0.5$ , should be identical with the maximum buffering capacity,  $\beta_M$ .

reversibly associated complex should lead to dissociation. With electrolytes, for example, and with antigen-antibody complexes, the phenomenon is a familiar one. Hussey and Northrop (5), working with trypsin and the inhibitory substance contained in plasma, observed that dilution resulted in dissociation and used this as evidence for the formation of a reversible complex acting in accordance with the mass action law. However, no work has come to our attention putting the dilution effect itself on a sound quantitative basis.

In diluting an enzyme-inhibitor mixture the specific enzyme concentration  $E'$  will always be changed to exactly the same degree as the specific inhibitor concentration  $I'$ , in other words, the ratio  $I'/E'$  (or  $I/E$ ) will be maintained constant. Thus, to represent dilution of such a mixture on Fig. 2 we travel from the original  $E'$  curve to the more dilute  $E'$  curve, but we must at the same time move a corresponding distance along the  $I'$  axis.

We will begin by considering the effect of dilution within zone C, where  $E' \geq 100$ . For example, to dilute from  $E' = 1000$  to  $E' = 100$ ,  $I'$  is necessarily also diluted 10 times, so that from a selected point on the curve  $E' = 1000$  we move to the left 1 log unit measured along the  $I'$  axis. It will be seen that having moved this distance horizontally to the left, we find ourselves on the curve  $E' = 100$  without having to move up or down, so that the inhibition  $\alpha$  remains unchanged. *The concrete meaning of this is that within zone C dilution has no effect whatever upon the fractional inhibition  $\alpha$ .*

To represent dilution in the region where  $E' \leq 0.1$ , we carry out the same steps as above, but the results are found to be quite different. For example, if we dilute 10 times in this region (e.g.  $E' = 0.1$  to  $E' = 0.01$ ) we must again move 1 log unit to the left on the  $I'$  axis (since  $I'$  is also diluted 10 times). However, the curve for  $E' = 0.01$  is practically identical with that for  $E' = 0.1$ , so that we must finally find ourselves on the same  $E'$  curve from which we began. This necessarily involves traveling up the curve and thereby ending with a smaller fractional inhibition  $\alpha$  than we started with. Since all values of  $E'$  smaller than 0.1 are represented by the same curve, it is characteristic of dilution in zone A that the change produced in  $\alpha$  is not influenced by initial or final specific enzyme concentrations, but only by the factor of dilution.

If we carry out the same steps in zone B we find that our travel to the left on the  $I'$  axis always carries us beyond the proper  $E'$  curve, so that we are forced, as in zone A, to travel up the curve and thereby change the value of  $\alpha$ . *In zone B, therefore, the fractional inhibition does change with dilution but the amount of change depends not only upon the factor of dilution, but also upon the initial and final  $E'$ .*

We have shown that in zones A and B dilution of an enzyme-inhibitor mixture results in dissociation so that the measurable inhibition  $\alpha$  is decreased, and that in zone C this does not occur. We have also pointed out that many enzyme systems operate in zone B and that others which may operate in zone

C *in vivo* are brought into zone B or even into zone A by dilution for experimental purposes. Therefore, since so many known enzyme-inhibitor systems are subject to the dilution effect, it will be necessary to place this effect upon a quantitative basis so that appropriate corrections may be made.

It would be a mistake to think that the errors arising from neglect of the dilution effect are small. As a matter of fact, they are so enormous as to invalidate conclusions based upon the application of experimental values of  $\iota$  at various dilutions to undiluted serum or other body fluids. It is also probable that often observed discrepancies between experimentally determined values of  $\iota$  and concomitant physiological responses may now be reconciled when the corrections for dilution are applied.

### *Practical Tests*

The magnitude of the dilution effect will be considered in a quantitative way below. We wish first to point out some useful tests based upon the zone behavior outlined above.

*1 Test for Presence of Inhibitor*—If no inhibitor is present, there is no inhibition, regardless of dilution, and the enzyme always works at its maximum velocity. This is shown in Fig. 2 by the fact that as  $I'$  approaches zero,  $\iota$  becomes zero for all the values of  $E'$ . This may seem rather obvious, but it is no less important, for failure to show direct proportionality between reaction velocity and enzyme concentration (provided that diffusion is not a significant factor) is strong evidence for the presence of a reversible inhibitor. Thus, in zones A and B, if such an inhibitor is present, the reaction velocity after dilution will be greater than direct proportionality would allow.

*2 Rough Test for Zone Behavior*—It has previously been mentioned that the slope of the experimentally determined inhibition curve at  $\iota = 0.5$  yields a rough indication of the value of  $E'$  and hence of the zone (see p. 568).

*3 Test for Zone A Behavior*—It will be recalled from Fig. 2 that for  $E' \leq 0.1$ ,  $\iota$  is determined solely by  $I'$ . Thus, if, and only if, a given total concentration of inhibitor produces the same inhibition at two different concentrations of enzyme, the system must be in zone A, at both enzyme concentrations.

*4 Test for Zone C Behavior*—It was shown above that only in zone C is the dilution effect absent. Thus, if, and only if, dilution of any mixture of enzyme and inhibitor produces no change in inhibition, the system must be in zone C.

### *Algebraic Representation of the Dilution Effect*

The magnitude of the dilution effect for any values of  $E'$  and  $I'$ , for any initial inhibition ( $\iota$ ), and for any factor of dilution, may best be determined algebraically. Although the graphical method given above is useful for visualizing what is going on, it does not afford the accuracy of an algebraic treatment.

Let equation 3B be rewritten in the form

$$I' = \frac{i_1}{1 - i_1} + i_1 E' \quad (5)$$

where  $i_1$  is the observed fractional inhibition, and

$E'$  is the concentration of enzyme in the reaction mixture where the observation is made.

Let  $N$  be a factor by which both  $I'$  and  $E'$  must be multiplied in order to reach the concentration at which the new inhibition,  $i_2$ , is to be calculated. Thus  $N > 1$  in going, for example, from diluted serum with an observed inhibition of  $i_1$  to undiluted serum with an inhibition of  $i_2$ . Conversely  $N < 1$  when it is desired to calculate the inhibition,  $i_2$ , in a system more dilute than the reaction mixture on which the determination is made. When the reactants are at  $N$  times their initial concentration, equation 3B becomes

$$NI' = \frac{i_2}{1 - i_2} + i_2 NE'$$

Dividing by  $N$

$$I' = \frac{1}{N} \frac{i_2}{1 - i_2} + i_2 E' \quad (6)$$

eliminating  $I'$  by combining equations 5 and 6,

$$\frac{1}{N} \frac{i_2}{1 - i_2} + i_2 E' = \frac{i_1}{1 - i_1} + i_1 E'$$

Solving for  $i_2$ ,

$$i_2 = \frac{1}{2} \left\{ \left[ \frac{1}{E'} \frac{i_1}{1 - i_1} + i_1 + \frac{1}{NE'} + 1 \right] - \sqrt{\left[ \frac{1}{E'} \frac{i_1}{1 - i_1} + i_1 + \frac{1}{NE'} + 1 \right]^2 - 4 \left[ \frac{1}{E'} \frac{i_1}{1 - i_1} + i_1 \right]} \right\}$$

and simplifying<sup>a</sup> the term under the radical,

$$i_2 = \frac{1}{2} \left\{ \left[ \frac{1}{E'} \frac{i_1}{1 - i_1} + i_1 + \frac{1}{NE'} + 1 \right] - \sqrt{\left[ \frac{1}{E'} \frac{i_1}{1 - i_1} + i_1 + \frac{1}{NE'} - 1 \right]^2 + \frac{4}{NE'}} \right\} \quad (7B)$$

<sup>a</sup> Let

$$\left[ \frac{1}{E'} \frac{i_1}{1 - i_1} + i_1 \right] = X \quad \text{and} \quad \left[ \frac{1}{NE'} \right] = Y$$

Then

$$[X + Y + 1]^2 - 4X = [X + Y - 1]^2 + 4Y$$



Equation 7B is a general solution for the inhibition at a new dilution when the original  $E'$ , the original inhibition ( $i_1$ ), and the dilution factor are known. Because it involves the difference of two terms of the same magnitude, calculation from this equation must be accurate to three decimal places, but otherwise the equation is not especially cumbersome.

It is of some interest to examine the limiting cases in this equation. It will be evident that when  $i_1 = 0$ ,  $i_2 = 0$ . When  $E' = \infty$ ,  $i_2 = i_1$  (7C). Both these results have been previously derived in this paper.

When  $E' = 0$ , evaluation of the equation becomes very difficult because the terms containing  $E'$  become infinite. However, the same steps used to derive equation 7B from equation 3B can be applied to equation 3A, which describes the zone where  $E' \doteq 0$ . It is then found that

$$i_2 = \frac{N i_1}{1 - i_1 + N i_1} \quad (7A)$$

This equation is valid for all systems within zone A and may be applied in place of the more complex equation 7B.

### *Plot of Dilution Effect*

A practical way of visualizing the dilution effect quantitatively is presented in Fig. 3. This figure is simply a plot based upon equation 7B, a value of  $E'$  being used which corresponds with our experimentally determined specific concentration of horse serum cholinesterase (see p. 578). This particular system was found to operate in zone B,  $E'$  being equal to 3.29 in undiluted serum. Arbitrarily selecting a number of values of inhibition in 22.2 per cent serum, corresponding values of  $i_2$  were calculated for each of several dilutions. The 22.2 per cent serum inhibitions were then represented as a straight line with slope = 1, and the various corresponding values of  $i_2$  plotted accordingly as abscissae.

To use this graph for dilution or concentration one simply travels to right or left on a horizontal line. The values of inhibition are read off directly from the abscissa. Thus, for example, an inhibition  $i = 0.8$  in serum becomes 0.5 at 22.2 per cent, 0.2 at 4.54 per cent, and 0.05 at 1.0 per cent. In this example the absurdity of concluding from a determination in 1.0 per cent serum that the undiluted serum was practically uninhibited needs no further emphasis.

It is perhaps best to think of the dilution effect in terms of the distortion of ranges of inhibition. To take the most extreme example, reference to Fig. 3 will show that the entire range from 0.1 to 0.9 in actual serum is represented at 1.0 per cent by the experimentally determined range 0.01 to 0.1. On the other hand, the whole experimental range 0.1 to 1.0 at this dilution is seen to represent the very small range 0.9 to 1.0 in actual serum. Similar but less serious distortions are observed at higher experimental concentrations.

These considerations make it plain that methods involving considerable dilution are less useful the greater the factor of dilution. In the example cited, for observed values of  $i$  between 0.01 and 0.1 experimental errors are magnified

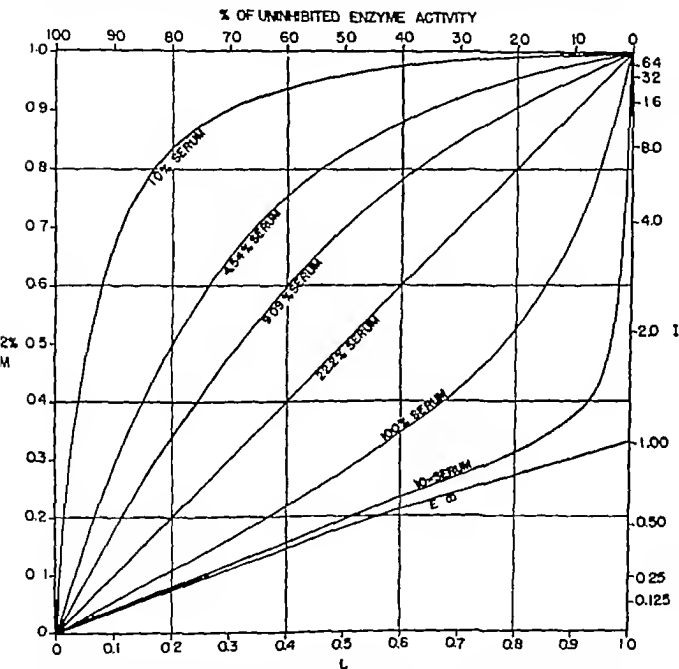


FIG. 3 Abscissa, fractional inhibitions  $i$  at various concentrations. Ordinate, left, corresponding inhibitions in 22.2 per cent serum.

Ordinate, right, ratios of total inhibitor to total enzyme concentrations. This ratio remains constant with dilution of any particular enzyme-inhibitor mixture.

tenfold when the appropriate conversion to serum inhibition is performed. At the same time, because observed values of  $i$  between 0.1 and 1.0 represent so small and comparatively unimportant a range of actual serum inhibitions, the major part of the method's usefulness is wasted.

The chief theoretical considerations and practical tests implicit in the concept of zone behavior of enzyme systems are summarized in Table I

TABLE I

Zone A	Zone B	Zone C	Remarks
$E' < 0.1$	$0.1 < E' < 100$	$E' > 100$	Values given are approximations where $\Delta s = 0.01$ . For exact boundaries as a function of $s$ , see Fig. 1
$I = K \frac{s}{1-s}$ (2A) Total = free $I' = \frac{s}{1-s}$ (3A)	$I = K \frac{s}{1-s} + sE$ (2B) Total = free + bound $I' = \frac{s}{1-s} + sE'$ (3B)	$I = sE$ (2C) Total = bound $I' = sE'$ (3C)	$E$ does not enter equations for zone A, nor $K$ in those for zone C. In zone C, inhibitor combines quantitatively with enzyme true for all values of $s$ reasonably below 1.0
$s$ produced by a given $I'$ is independent of $E'$	$s$ is dependent on both $I'$ and $E'$	$s$ is dependent on both $I'$ and $E'$ . $I'$ required to produce a given $s$ is directly proportional to $E'$	Can be used as criteria for zones A and C
Because $E$ does not appear in equations, no definite value can be assigned to it by any method involving measurement only of reaction velocities and of $I$ . $K$ can be evaluated, $E'$ cannot	Both $E$ and $K$ appear and can be assigned definite values, so can $E'$	Because $K$ does not appear in equations, it cannot be evaluated by any method involving only reaction velocities and $I$ . $E$ can be evaluated, $E'$ cannot	The use of the terms $I'$ and $E'$ in equation 3C does not permit evaluation of $K$ , since $K$ cancels out of both sides of the equation
Michaelis equation applies	Michaelis equation does not apply	Michaelis equation does not apply	Error in the determination of $K$ by measuring the concentration of $I$ when $s = 0.50$ rapidly becomes great when $E'$ exceeds 0.1
$\frac{ds}{d \log I'} = 0.575$ , when $s = 0.50$ for all values of $s$	$0.575 < \frac{ds}{d \log I'} < 1.51$ , when $s = 0.50$ for all values of $s$	$\frac{ds}{d \log I'} = 1.51$ , when $s = 0.50$ for all values of $s$	Can be used as criteria for zones and for rough evaluation of $E'$ in zone B
Dilution effect present and independent of $E'$	Dilution effect present and varies with $E'$	Dilution effect absent; $s$ does not change on dilution	Can be used as a criterion of zone C
$s = \frac{N s_1}{1 - s_1 + N s_1}$ (7A)	$s = f(s_1, E', \text{ and } N)$ see equation 7B	$s = s_1$ (7C)	

## EXPERIMENTAL

To test the validity of any hypothesis it is sufficient to test any one function that includes all the assumptions implicit in the original hypothesis. The

dilution equation provides such a test, and will be shown to describe the behavior of mixtures of physostigmine and cholinesterase with satisfactory accuracy over a wide range of enzyme concentration.

### *Determination of $E'$*

(a) *Method*—Determinations of the cholinesterase activity of unpurified horse and dog serum for the calculation of the dissociation constant of the enzyme inhibitor complex and the molar concentration of enzyme centers were done by the method of Friend and Krayner (6). The final reaction mixture contains 22.2 per cent serum and 2.7 per cent (0.12 molar) acetylcholine bromide in bicarbonate Ringer solution at pH 7.4 and 38°C, equilibrated continuously with vigorous mechanical stirring against a 5 per cent carbon dioxide—95 per cent nitrogen gas mixture. An equimolecular amount of carbon dioxide is displaced by the production of acetic acid from acetylcholine during its hydrolysis by the enzyme. Exactly 1.00 cc. of serum is added to 3.0 cc. of bicarbonate Ringer and equilibrated for 15 minutes; after 0.50 cc. of 24 per cent acetylcholine bromide in Ringer solution is added and equilibration continued for 3 minutes more, a 1 cc. aliquot is removed, and another aliquot is removed exactly 20 minutes later. The difference in the carbon dioxide content per liter of the two samples done by the Van Slyke manometric method equals the millimoles of acetylcholine hydrolyzed per liter of reaction mixture per 20 minutes.

Expressed in terms of millimoles of acetylcholine hydrolyzed per liter of 100 per cent serum per hour, the average titer of horse serum by this method was 200 mm/liter/hr., with a range of 160 to 240 in samples from different animals. The average value for dog serum was 120 mm/liter/hr., with a range of 70 to 160. In the work with horse serum described below, a pooled batch of sterile serum with a titer of 202 mm/liter/hr. was used. This value was found to remain constant for a number of weeks even in serum held at 38°C without sterile precautions.

Measurement of the fractional inhibition produced by a known concentration of physostigmine salicylate was carried out by dissolving a known amount of drug in the 3.00 cc. of bicarbonate Ringer solution used to dilute the serum before equilibration. The inhibitor was thus in contact with the enzyme for 18 minutes before the start of the 20 minute period of measurement. This order of adding the reactants is important inasmuch as consistently smaller values of  $i$  are obtained if the inhibitor is added *after* the substrate. (See discussion on competition, p. 583.)

(b) *Results*—Experimental points for the fractional inhibition produced in 22.2 per cent horse serum by various molar concentrations of physostigmine in the reaction mixture are plotted in Fig. 4. These define the whole curve of inhibition,  $i$ , versus the logarithm of the molar concentration of inhibitor,  $I$ ,

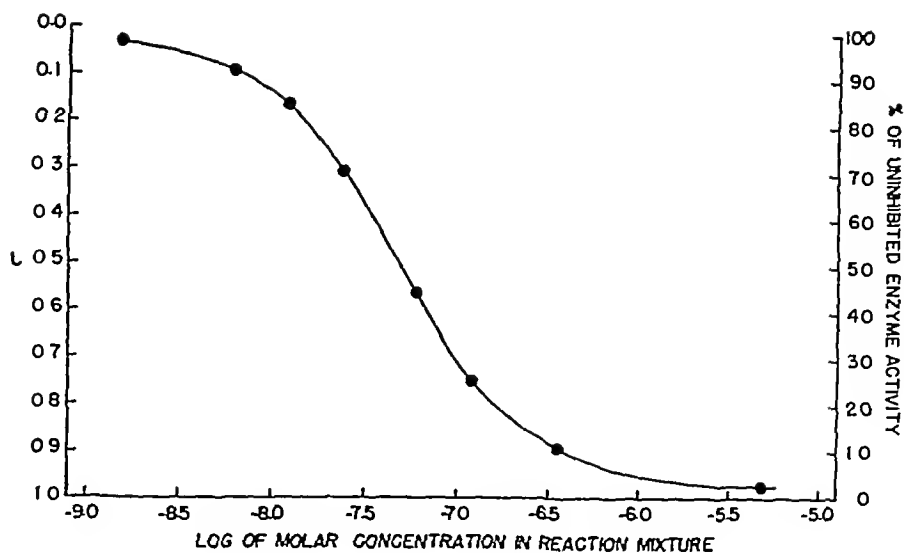


FIG 4 Fractional inhibition of horse serum cholinesterase as a function of physostigmine salicylate concentration. Enzyme concentration in reaction mixture,  $E = 2.7 \times 10^{-8}$  molar, and  $E' = 0.73$  (see Fig 5). This is a specific example of the generalized curves depicted in Fig 2.

TABLE II

A	B	C	D	E
$i$	Observed $I \times 10^3$ (molar)	$\log_{10} I$ (molar)	$I/i \times 10^3$	$1/(1-i)$
*0.03	*0.15	-8.82	5.0	1.03
0.05	0.32	-8.50	6.4	1.05
*0.09	*0.62	-8.21	6.9	1.10
*0.16	*1.23	-7.91	7.7	1.19
0.20	1.59	-7.80	7.9	1.25
*0.30	*2.45	-7.61	8.2	1.43
0.40	3.63	-7.44	9.1	1.66
0.50	5.12	-7.29	10.2	2.00
*0.56	*6.03	-7.22	10.8	2.28
0.60	7.25	-7.14	12.1	2.50
0.70	10.0	-7.00	14.3	3.33
*0.75	*12.2	-6.92	16.3	4.00
0.80	17.0	-6.77	21.2	5.00
*0.89	*36.3	-6.44	40.7	9.10
*0.97	*490	-5.31	505	33.3

\* Indicates observed values, other values of  $i$  and  $I$  interpolated from plot of observed values of  $i$  and  $I$  (Fig 4).

within very close limits, and the values are typical of other runs. The observed values and additional points interpolated graphically from Fig 4 are tabulated in columns A, B, and C of Table II.

From the values of  $\log I$  where  $z = 0.3$  and  $0.7$ , the slope  $di/d \log I$ , in this segment is found to equal  $0.66$ . Since this value lies between the limits  $0.575$  and  $1.151$ , the function is at least compatible with reaction B (see p. 568 above). Substituting the value  $di/d \log I = 0.66$  in equation 4,<sup>9</sup> the preliminary figure for  $E'$  turns out to be approximately  $0.7$ . This indicates that the reaction mixture probably lies in the lower half of zone B. A more rigorous method for determining  $K$  and  $E$ , and hence  $E'$ , is therefore in order. The method had been applied to cholinesterase and prostigmine by Easson and Stedman (7)

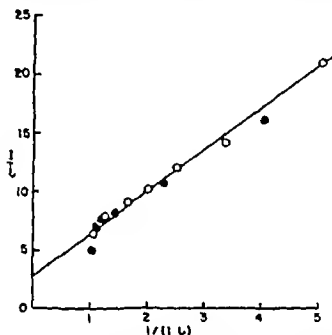


FIG. 5. Graphic method of determining  $K$  and  $E$  (molar) for serum cholinesterase-physostigmine system by plotting  $I/i \times 10^3$  against  $1/(1-i)$ . Values are tabulated in Table I.  $E$  = ordinate intercept of the straight line =  $2.7 \times 10^{-3}$ .  $K$  = slope of the straight line =  $3.7 \times 10^{-3}$ . ● = observed values. ○ = interpolated values.

Let equation 2B be divided by  $i$ . Then,

$$\frac{I}{i} = K \frac{1}{1-i} + E \quad (8)$$

This equation is linear with respect to  $I/i$  and  $1/(1-i)$ . A plot having these terms as ordinate and abscissa respectively will therefore yield a straight line if the observed values are compatible with the assumptions upon which the equation is based: the slope will numerically equal  $K$ , and the ordinal intercept will equal  $E$ . Calculated values of  $I/i$  and  $1/(1-i)$  appear in columns D and E of Table II and are plotted in Fig. 5.

Since  $i$  and  $(1-i)$  appear as the denominators of these two terms, a small absolute error in  $i$  will have the greatest numerical effect on  $I/i$  when  $i \rightarrow 0$ ,

\* Equation 4 defines the term  $di/d \log I'$ . However, since  $I$  differs from  $I'$  by a constant  $K$ , the expression for slope used here will be equal to the term in equation 4.

and on  $1/(1 - z)$  when  $z \neq 1$ . For these numerical reasons, and because experimental accuracy of the determination of  $z$  also falls off at the extremes, this method is most practical for values of  $z$  between 0.2 and 0.8.

The points in Fig. 5 are seen to lie on a straight line, from the slope and intercept of which it is found that  $K = 3.7 \times 10^{-8}$  and  $E = 2.7 \times 10^{-8}$  molar, so that  $E' \approx E/K \approx 0.73$ . This value agrees with the estimate obtained by use of equation 4. It is, however, a more rigorous test of the compatibility of the data with equation 2B since it embraces a larger segment of the whole curve. The value of  $E'$  obtained by this method places the system cholinesterase-physostigmine in horse serum within zone B ( $E' = 3.29$  in undiluted serum). We should therefore expect on theoretical grounds that the dilution effect ought to be demonstrable in this system. The actual correspondence between theory and experiment may now be presented.

### *Dilution Effect*

(a) *Method*—For determination of the effect of diluting various mixtures of enzyme and inhibitor, the Warburg apparatus was used (method of Ammon (8)), since this method permits measurement of a wide range of reaction velocities. The temperature, pH, order of addition of reactants, timing, and concentrations were substantially the same as with the method of Friend and Kraye. Satisfactory agreement is obtained with uninhibited serum by the two methods when reduced to terms of millimoles acetylcholine hydrolyzed per liter of 100 per cent serum per hour. Measurements were made over the period from 3 to 23 minutes after addition of substrate, except in the case of 22.2 per cent serum (reaction mixture concentration) when, because of the high reaction velocity, 3 and 13 minute readings had to be used.

(b) *Results*—In the absence of inhibitor the velocity of acetylcholine hydrolysis at various dilutions was approximately proportional to the serum concentration. A slight tendency for the velocity to increase relative to serum concentration was noted at the greatest dilutions, but this was probably on a basis of less  $\text{CO}_2$  retention than at greater concentrations of serum.

The experimental results are summarized in Table III. Each horizontal row represents the inhibition in a single enzyme-inhibitor mixture, determined at four different dilutions (*i.e.*,  $I/E$  held constant). Observed values are in bold-face type, and for each such value are calculated (from equation 7B) the corresponding points at every other dilution and in undiluted serum. Thus in each horizontal line are found one observed value and four corresponding values.

For a given mixture at a particular dilution the observed and calculated values are seen to agree quite satisfactorily, with two or three exceptions at the extremes of dilution and inhibition. The best set of values is that for  $I/E = 10.7$ , where the inhibition ( $z$ ) varies from 0.85 to 0.19 if the mixture is diluted

from 22.2 per cent to 1.0 per cent of its initial concentration, while in undiluted serum as it would exist in the experimental animal, the inhibition is 0.96

TABLE III  
Effect of Dilution on Inhibition,  $i$   
 $E'$  in undiluted serum = 3.29

Serum concentration in per cent of undiluted serum	1.00	4.54	9.09	22.2	100
$I/E$					
1 1	0 020	0 08	0 14	0 24	0 43
	0 020	0 07	0 12	0 21	0 39
	0 015	0 06	0 10	0 18	0 33
	0 025	0 10	0 17	0 30	0 53
1 8		0 09		0 24	0 43
		0 11		0 30	0 53
2 1	0 02	0 08	0 14	0 24	0 43
	0 05	0 18	0 28	0 46	0 74
	0 05	0 17	0 28	0 46	0 74
	0 05	0 19	0 30	0 48	0 76
9 1		0 38		0 72	0 91
		0 33		0 69	0 90
10 7	0 19	0 50	0 67	0 83	0 96
	0 21	0 64	0 70	0 85	0 96
	0 20	0 52	0 68	0 84	0 96
	0 21	0 54	0 70	0 85	0 96
21 5	0 22	0 55	0 71	0 86	0 95
	0 37	0 72	0 84	0 93	0 98
	0 30	0 64	0 79	0 90	0 97
	0 30	0 64	0 79	0 90	0 97
215	0 78	0 92	0 97	0 99	0 998
	0 56	0 88	0 94	0 97	0 995
	0 50	0 82	0 90	0 96	0 99
	0 50	0 82	0 90	0 96	0 99

Although these experimental data do not constitute a perfect verification of our theoretical premises, we believe that they are sufficiently impressive to serve as strong corroborative support for the validity of equation 7B, and consequently of the zone concept in general. Apart from all theoretical considerations, the practical corollary of equation 7B has been adequately proven that it is unwarranted to assume that determinations of the state of an enzyme



inhibitor system *in vitro* give an accurate picture of the state of that system in the circulating serum. It is now possible for the first time to calculate these serum enzyme inhibitions *in vivo* and furthermore to compare the results of different investigators, who may use various methods involving a variety of dilutions of the enzyme-inhibitor mixture.

### *Turnover Number*

Since under the experimental conditions described, 1 liter of uninhibited reaction mixture hydrolyzes 15 millimoles of acetylcholine in 20 minutes, and since  $E = 2.7 \times 10^{-8}$  molar, it follows that each active enzyme center breaks down 450 molecules of acetylcholine per second. This turnover number is  $\frac{1}{3}$  that reported by Easson and Stedman (7), and our dissociation constant is 3 to 4 times that reported by Roepke (9) working on serum cholinesterase largely freed of inert protein by the method of Stedman and Stedman (10). Roepke, moreover, noted that 3 to 4 times as much physostigmine was necessary to produce a given inhibition in crude serum as in the purified product, probably because of fixation of inhibitor by inert protein.

Inspection of equation 2B shows that a false high value of  $I$ , resulting from such a circumstance, would raise the apparent value of  $E$ , and account for the direction of divergence in our figure for the turnover number. Nevertheless, we wish to emphasize that our values of  $K$  and  $E$ , and the conjugate values of  $i$  and  $I$  so obtained, fit the results within the limits of experimental error, and that the values obtained with purified enzyme are inapplicable to crude serum, which, after all, is what circulates in the vessels of the experimental animal.

## DISCUSSION

### *Evolution of the Concept of Zone Behavior*

Since there is nothing in the foregoing analysis of the union of one enzyme center with one molecule of substrate or inhibitor that is not implicit in the law of mass action, the question arises why the differences in zonal behavior and the dilution effect have not previously been pointed out and put to use. The answer perhaps lies in the formulation itself. It has been shown that the enzyme systems that have most frequently been used as prototypes for general discussions of enzyme kinetics ordinarily behave like monomolecular reactions (are pseudomonomolecular) under the conditions of measurement, *i.e.*, they lie in zone A. The cholinesterase-physostigmine system used as a prototype in this paper was examined at enzyme concentrations that place the system in a zone where the monomolecular function no longer adequately describes the results obtained. Easson and Stedman (7), recognizing this fact in their paper on the kinetics of a similar system, correctly used the full second order function but did not carry the analysis to its ultimate conclusion.

Although a first order kinetic function has the advantage of simplicity, its application to a true second order reaction such as the reversible union of an

enzyme and its inhibitor may lead to serious error under conditions where the concentration of the second reactant becomes of importance. It therefore seemed sounder to us to set up the function covering the reversible union of two reactants to form a complex, and then to attempt to establish rigorous limits within which the use of the monomolecular function causes less than some definite and negligible error. It has become apparent in the course of this analysis that such systems show not two, but three zones of behavior: a zone adequately described by the monomolecular function (zone A), a zone where the full bimolecular function must be used (zone B), and finally a zone in which the reactants will appear to combine with each other stoichiometrically according to the law of definite proportions, although the reaction is still fully reversible (zone C).

### *Biological Significance of Zone C Behavior*

The likelihood that many enzymes, at the points where they function in tissue, are highly concentrated and therefore in zone C with respect to their substrates or to inhibiting substances has already been mentioned. It follows from the stoichiometric behavior of the reactants in this zone that if a biological effect is found to be a linear function of the dose or concentration of an inhibitor, one need not necessarily conclude that the reaction is irreversible, but only that, if reversible, the reactant with which the inhibitor combines has a specific concentration high enough to place the system in zone C.

There is another conclusion which should prove to have widespread practical application in experimental pharmacology. Since in zone C inhibition ( $i$ ) is equal to the ratio  $I/E$  without reference to  $K$ , and since it is precisely the dissociation constant  $K$  that distinguishes one inhibitor from another in the effect upon a given enzyme, it follows that for any enzyme in tissue at fairly high concentration, *all reversible inhibitors should produce the identical effect*, provided only that the various values of  $K$  are all of such magnitude that the system remains in zone C. A simple example will illustrate the point. Let us consider, as Nachmansohn (11) has shown, that cholinesterase in the body is very highly concentrated at the motor end-plates, for example,  $E = 10^{-4}$ . We will now assume two inhibitors, one, like physostigmine, with  $K$  about  $10^{-8}$ , the other with  $K$  about  $10^{-6}$ . Having determined the dissociation constants in dilute serum, and having observed the greater potency of physostigmine under such conditions, we would naturally assume that the biological response to this drug would be far greater, perhaps a hundred times as great. We now see, on the contrary, that since both values of  $K$  are such as to leave the system within zone C there should be no demonstrable difference in the biological action of the two drugs. If this prediction is sound, we must conclude that with drugs of this type it is futile to seek increased potency except as such efforts are directed toward the problems of toxicity, distribution, inactivation, etc.

### *Definition of Zone Boundaries in Terms of I*

Since reaction  $B$ , denoting the reversible union of two reactants, is symmetrical with respect to both reactants, the question arises why the specific enzyme concentration,  $E'$ , rather than the specific inhibitor concentration,  $I'$ , has been used to define the zone boundaries. The answer lies in the fact that the choice of variables in the function relating the concentrations of the reactants and their complex (equation 3B), is determined by what quantities are conveniently measurable and what quantities it is desirable to calculate by means of these equations. It is usually convenient to measure the total concentration of inhibitor employed, and it seems logical to employ a term to denote the total concentration of enzyme,  $E$ . Since in most enzyme work the fraction of total enzyme,  $\tau$ , that is in the combined form is of primary interest, it is both logical and convenient to introduce this asymmetrical term into the kinetic equations. However, under circumstances where interest was focused upon the fraction of total substrate or inhibitor that is combined with enzyme, there would be equal justification for exchanging the places of  $E$  and  $I$  and letting  $\tau$  now represent  $I_{\text{combined}}/I_{\text{total}}$ . The zone boundaries would then be determined by the specific inhibitor concentration,  $I'$ , whose numerical boundary values will be the same as those for  $E'$ . The application of this concept to an analysis of the rate of destruction of acetylcholine at the nerve ending in relation to the refractory period of the nerve will be treated in a note to be published later.

### *Extension of the Analysis to More Complex Systems*

It has been emphasized that this analysis can be applied to any reaction of the form  $A + B \rightleftharpoons AB$ . This would include many antigen-antibody reactions, weak acid or base dissociations, solubility products, etc., as well as certain enzyme systems.

In considering the case of an enzyme combining with substrate alone, we must, of course, make the customary "steady state" assumptions for reaction  $A$  ( $E + X \rightleftharpoons EX \rightarrow E + Sp$ ), the concentration of  $EX$  remaining constant and that of  $X$  not changing appreciably during the reaction. The combined form of the enzyme is now active, so that we may call  $(ES)/E$  the *fractional activity*, and designate it by  $a$ , which will then be substituted for  $\tau$  in all the equations. With this minor reorientation, the analysis applies to uninhibited enzyme reactions, which should display the same zone behavior and dilution effects already demonstrated for inhibited systems.

For reactions of a higher kinetic order, it will be desired to generalize the foregoing analysis to apply to the case where one molecule of a reactant combines reversibly with  $n$  molecules of a second reactant ( $E + nI \rightleftharpoons EI_n$ ). It can be shown by the same steps used to derive equation 3B that

$$I' = \sqrt[n]{\frac{\tau}{1-\tau}} + nE'$$

where  $E' = E/\sqrt{K}$ , and  $I' = I/\sqrt{K}$ . This is merely a more generalized form of equation 3B, and the same analysis can be applied to derive zone boundaries and the equations describing the dilution effect. All statements applying to the zones will still apply. In general, as  $n$  increases the boundaries of zone B will approach each other. Equation 7A describing the dilution effect in zone A becomes

$$t_2 = \frac{N^n t_1}{1 - t_1 + N^n t_1}$$

An increase in  $n$  very markedly increases the change of inhibition with dilution, an effect that can be experimentally measured. This function thus provides a very sensitive criterion for testing the number of molecules of inhibitor, for instance, that combine to form an inactive complex.

### *Limitations of This Analysis*

The analysis developed in this paper is incomplete in at least one important respect, as a consequence of which important limitations are placed upon some of our conclusions. As already mentioned at the outset, the assumption of a reversible reaction between enzyme and inhibitor makes it mandatory also to assume that the addition of substrate for purposes of determination results in the displacement of a certain number of inhibitor molecules from combination, provided only that inhibitor and substrate combine with the same active center of the enzyme molecule. Thus substrate addition must cause a decrease in  $s$ . That the effect can be noticed even within the initial 20 minute period required for the determination was indicated on p. 575. We have pointed out that the quantitative significance of this competition effect will depend upon the constants for any particular system. A completely valid treatment, however, should be based upon the final equilibrium attained between enzyme, inhibitor, and substrate, rather than upon the first two alone. More accurate values of  $K$  and  $E$  could then be obtained, for if competition is significant within the first 20 minutes the curve of Fig. 4 may no longer be interpreted on the basis of reaction B and equations 2B and 3B. Work placing the competition effect on a sound theoretical and experimental basis is now in progress in this Laboratory and will be the subject of a subsequent publication.

Despite its inadequacies, the present *non-competitive* treatment nevertheless applies *fully* (1) to the case of an inhibitor which reversibly inactivates an enzyme by combining at a different point from the substrate or by causing physical alteration of the enzyme molecule, (2) to the case of uninhibited enzyme-substrate and other comparable systems, provided the substrate concentration does not change appreciably during the course of the reaction. The present treatment applies *practically* to the case where competition is not significant compared with the function being measured. It is on this basis that

we feel justified in illustrating our method and confirming the dilution effect by means of the cholinesterase-physostigmine system, which has in the past been considered practically non-competitive

#### SUMMARY

1 The kinetics of the reversible combination of one enzyme center with one molecule of a substrate or inhibitor is treated as a true bimolecular instead of a pseudomonomolecular reaction. The general equations describing such a reaction are presented and analyzed algebraically and graphically.

2 A new term, "specific concentration," is introduced to denote the concentration of reactants in units equal to the dissociation constant. Its use makes the kinetic equations universally applicable to all reversible systems of the given type.

3 It is shown that such a system exhibits three "zones" of behavior. Each zone is characterized and shown to exhibit significant differences in the function relating the concentrations of the components of the system at equilibrium. The zone boundaries are rigorously defined in terms of the specific enzyme concentration, for the mathematical error tolerable with a given experimental accuracy, and approximate boundaries for practical use are proposed.

4 The classical treatment of enzyme kinetics is shown to be a limiting case valid only for low specific enzyme concentrations (zone A) and to be inapplicable in a number of systems whose dissociation constants are very small or whose molar enzyme concentrations are very great, and in which, therefore, the specific enzyme concentrations are large. See Table I for a summary of zone differences.

5 In an enzyme system containing substrate or inhibitor, dilution before determination of reaction velocities is shown to be a crucial operation, entailing large changes in the fraction of enzyme in the form of a complex. The changes in fractional activity or inhibition with dilution are shown to be a function of specific enzyme concentration, the dilution factor, and the fraction of enzyme initially in the form of complex. Equations are given permitting the calculation of the state of the system at any concentration. The errors introduced into physiological work by failure to take the dilution effect into account are pointed out.

6 Experimental data are presented showing that the system composed of serum cholinesterase and physostigmine behaves as predicted by the dilution effect equations.

7 Two other conclusions of practical pharmacological importance are drawn from the theory of zone behavior.

(a) The finding that a biological response is a linear function of the dose of a drug does not necessarily mean that the reaction is irreversible, but only that if reversible, the reactant with which the drug combines has a high specific concentration.

(b) If a tissue enzyme has a high specific concentration, all reversible inhibitors will be equally potent in combining with it, regardless of their relative potency in dilute systems provided only that their dissociation constants are within certain broad limits.

8 It is shown how the type of analysis here applied to bimolecular reactions can be applied *in toto* to systems of the type  $E + nX \rightleftharpoons EX_n$ , where  $n$  molecules of substrate or inhibitor unite with one enzyme center. The zone boundaries and the magnitude of the dilution effect change with  $n$ , but the general characteristics of the zones are the same for all values of  $n$ .

9 Since the analysis is based only on mass law assumptions, it is applicable to any system that is formally analogous to the one here treated.

We wish to express our gratitude to Dr Otto Kraye, who made available the facilities of his laboratory and under whose patient guidance this work was brought to completion. Thanks are also due Dr John T Edsall for generous advice and criticism during preparation and revision of this paper.

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